Effects of mixed lineage leukaemia, the human homologue of Trithorax, and its leukaemic fusion proteins in Drosophila melanogaster

Thesis

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Effects of Mixed Lineage Leukaemia, the human homologue of Trithorax, and its Leukaemic fusion proteins in *Drosophila melanogaster*

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induces faster cell cycle initiation.

-Abbreviations-

a.a.	amino acid
ALL	Acute Lymphoblastic Leukaemia
AML	Acute Myeloid Leukaemia
ANT-C	Antennapedia complex
ash1/ ash2	absent, small and homeotic discs 1/ 2
ATP	adenosine triphosphate
Bmi-1	B-cell specific Mo-MLV Insertion region 1
bp	base pair
β-gal	β-galactosidase
brg1	brahma related protein 1
Brm	brahma
BSA	bovine serum albumin
BX-C	Bithorax complex
CBP	CREB binding protein
cDNA	complementary DNA
ddH_20	double-distilled water
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E(z)	Enhancer of Zeste
E.coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
eed	embryonic ectoderm development
Enx1/ Enx2	E(z) homologue 1/ 2
esc	extra sex combs
ETP	Enhancer of *Polycomb* and *trithrorax*
g	gram/ gravity
H1	histone H1
H2A	histone H2A

H2B	histone H2B
H3	histone H3
H4	histone H4
HCl	hydrochloric acid
HDAC	histone deacetylase complex
HMGI (Y)	high mobility group I (Y)
HOX	homeobox gene
HP1	heterochromatin protein 1
HPC2	human Polycomb 2
Hsp70	heat-shock protein 70
IAA	indoleacrylic acid
ini1	integrase interactor 1
snf5	sucrose non-fermenting gene 5
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilobase
KCl	potassium chloride
kDa	kilo Dalton
LB	Luria-Bertani bacterial medium
M	molar
Mel-18	B16 melanoma 18S mRNA transcript
µg	microgram
mg	milligram
mM	millimolar
Mll	Mixed Lineage Leukaemia
mor	moira
mPc2	mouse Polycomb 2
Mph1 (rae28)	mouse polyhomeotic 1
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
ng	nanogram
NH_4HCO_3	ammonium carbonate
nmol	nanomol
NP-40	Nonidet P-40

O.D.	optical density
ORC	origin recognition complex
PAGE	poly-acrylamide gel electrophoresis
PBS	phosphate-buffered saline
pc	polycomb
PcG	Polycomb group
PcG-i	Polycomb group- initiating
PcG-m	Polycomb group- maintenance
PCR	polymerase chain reaction
PFA	para-formaldehyde
PLL	poly-L-lysine
ph	polyhomeotic
PHD	plant homology domain
pho	pleiohomeotic
PMSF	phenyl methyl sulphonyl fluoride
PRC1	Polycomb Repressive Complex 1
PRE	Polycomb response element
psc	posterior sex combs
Rb	retinoblastoma
Rfc4	Replication factor C 4
RNA	ribonucleic acide
rpm	revolutions per minute
scm	sex comb midleg
SDS	sodium lauryl sulfate
SET	Su(var)3-9, E(z), Trx
snr1	Snf5-related 1
su(var)3-9	suppression of variegation 3-9
SWI/SNF	switch/ sucrose non-fermenting gene (yeast)
TAC1	Trithorax Acetylation Complex 1
TRE	trithorax response element
Tris	tris(hydroxymethyl)aminomethane
trx	trithorax
trxG	trithorax group

Tween-20	polyoxyethylene sorbitan-20
V	volt
v/ v	volume to volume
°C	degree Celsius

Amino acids

A	alanine	M	methionine
C	cysteine	N	asparagines
D	aspartic acid	P	proline
E	glutamic acid	Q	glutamine
F	phenyalanine	R	arginine
G	glycine	S	serine
H	histidine	T	threonine
I	isoleucine	V	valine
K	lysine	W	tryptophan
L	leucine	Y	tyrosine

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Finally, I am thankful for the support my family has given me throughout the past and will, without a doubt, give in the coming years.

I leave Germany with a final thought: dampfnudel.

-Abstract -

Mixed Lineage Leukaemia (MLL) refers to the protein implicated in translocating with over 30 partner genes to form fusion proteins that lead to the development of acute leukaemia. MLL mediated leukaemia predominantly afflicts children under 5 years of age or younger, and is associated with very poor survival rates. Although MLL shares limited homology with the *Drosophila* Trithorax (TRX) protein, both MLL and TRX are speculated to alter gene expression profiles by modulating higher order chromatin structures.

The work presented here introduces *Drosophila melanogaster* as a supplementary model system for understanding how MLL leukaemic fusion proteins can contribute to leukemogenesis. The expression in the *Drosophila* system of the two most widely generated fusion proteins in leukaemic patients, MLL-AF9 and MLL-AF4, has defined pathway(s) through which the proteins may interfere with to induce fly lethality. Both fusion proteins were found to affect higher order chromatin structure by modifying the methylation pattern of histone H3 at lysine 9. Modified higher order chromatin modulates pathways targeted by specific MLL fusion protein, suggesting that the C-terminal portion of the fusion proteins triggers this step in cell cycle deregulation. Finally, the finding that late *trx* lethals are also associated with similar modified higher order chromatin structures displayed by MLL fusion proteins uncovered an additional role for TRX in the late development of *Drosophila*.

The results provide guidance in understanding why so many PcG and trxG members cause cancer when mutated, and suggest the existence of additional and distinct functions by PcG and trxG proteins at late development. The expression of MLL fusion proteins in *Drosophila* unveils relevant and concrete pathways through which particular MLL fusion proteins may act to prevent proper cell cycle progression. Put simply, this study links the targeting by MLL fusion proteins to TRX functions required for completion of late *Drosophila* development.

CHAPTER 1

Introduction

1.1 The emergence of epigenetics

Throughout evolution, the essential mechanisms involved in generating complex structures from a small number of undifferentiated cells have been conserved (see Section 1.2). The failure to properly execute these conserved pathways prevents the completion of embryonic development. Although epigenetic phenomena, the regulation of gene expression independent of DNA sequence, have been described for many decades, the accumulation of data demonstrating the importance of epigenetics on proper development was only very recently revealed.

A convincing piece of evidence supporting the role of epigenetics in development comes from studies involved in nuclear transfer technology. It was observed that cloning of animals by the transfer of isolated nuclei from somatic cells into enucleated oocytes were associated with high level of embryonic lethality, whereas those that survived, often acquired respiratory and circulatory problems and showed increase in birth weights (Wilmut et al., 1997; Kato et al., 1998; Baguisi et al., 1999; Onishi et al., 2000; Polejaeva et al., 2000). These defects have been shown in part to be due to unstable gene expression of genes that have not acquired proper imprinting (Humphreys et al., 2001). The widespread dysregulation of genes in survived cloned animals, demonstrates that mammalian development appears to allow a certain margin of epigenetic abnormalities. The accumulation of epigenetic aberrations affecting a number of genes higher than the tolerated threshold, results in lethality, as observed with the majority of the cloned animal cases (reviewed in Rideout et al., 2001).

In this respect, it is not surprising that mutations in several epigenetic pathways are also correlated with cancer, namely defects in cell homeostasis. During the past decades, experiments directed at unravelling the mechanisms associated with abnormal cell proliferation have resulted in the generally accepted notion that aberration of at least two major pathways are required for a cell to behave cancerous. The first involves desensitizing the cell, whereas the second introduces modified mitogenic factors that allow cell proliferation (reviewed in Hannah and Weinberg, 2001). The recent finding of tumor inducing cell lines generated by expressing SV40 large-T antigen, the telomerase catalytic subunit, and H-Ras oncoprotein in human mammary epithelial cells (HMECs), support the above model, as it illustrates that compromising the defence system

of a cell, in conjunction with the introduction of a mitogenic factor, do lead cells to uncontrolled proliferation (Elenbaas et al., 2001). The observation that the microenvironment surrounding transformed cells influences the ability of these cells to survive and to invade normal tissue, reinforces previous ideas that external factors greatly impact the stability of cells (Elenbaas et al., 2001). Furthermore, the recent demonstration that inactivation of a "nodal" or central point of a pathway is required to deregulate the cell cycle speaks for the redundancy of common pathways in mammalian cells (Chan et al., 2000). Finally, cell type has been shown to be a strong factor in determining which cells can be transformed. The report that epithelial cells as opposed to fibroblasts can accumulate mutations more easily, partly unravels why breast cancer is highly prevalent (Romanov et al., 2001).

Considering the points mentioned above, it becomes clear that cancer cells usually have compromised self-defense systems and proliferate in an unchecked manner. Given the abundance of different mutated signalling pathways in different cancerous cell types (reviewed in Hannah and Weinberg, 2001), it is likely that mutation of the basic gene expression regulation system, responsible for controlling the response to signalling pathways, as well as for maintaining the most crucial part of any cell, is likely to underlie the cancerous phenotype. This basic regulation system comprises the factors that control chromatin accessibility and gene expression. Several mechanisms that regulate gene expression have been described. Among them, epigenetics has emerged exponentially within the past few years. Although the molecular mechanisms by which epigenetics functions remain elusive, it is increasingly clear that epigenetics plays an important role in the biology of cancer, extending the influence of epigenetics beyond embryonic development, and into development after birth.

1.2 Conservation of developmental building blocks

The coordination of differentiation and proliferation for a small population of cells is central for the development of an organism. In insects and in mammals, mechanisms governing this process have been evolutionarily conserved.

The repeated, identical segments present in the *Drosophila* embryo, orginate from a small set of founder cells. Each segment is defined by a unit of cell lineage. The array of embryonic

segments subsequently diversifies to give rise to different structures, and is maintained in the same organisation as the adult body parts. Within each embryonic segment, or parasegment, two groups of founder cells are destined to either make the anterior or the posterior compartment of an adult segment. Consequently, the two founder cells within one embryonic parasegment give rise to the posterior compartment of an adult segment and to the anterior compartment of the next segment.

The definition of compartment has been postulated to be specified by selector genes acting on two sets of founder cells. The unique combination of selector gene expression in each founder cell population is associated with a particular type of compartment. According to the hypothesis, mutation in a selector gene results in an entire transformation of a given body compartment to another (Morata and Lawrence, 1975; Garcia-Bellido, 1975; Lawrence and Morata, 1994; Mann and Morata, 2000). Importantly, the faithful expression of selector genes throughout development is required for the determined state, indicating that selector gene expression must be maintained over many cell cycle generations.

The homeotic clusters were determined to be selector genes since mutations in any of the gene clusters resulted in entire compartment transformation. The *Drosophila* homeotic cluster comprises of two complexes, the antennapedia complex (ANT-C) and the bithorax (BX-C) complex. The five genes belonging to ANT-C and the three genes corresponding to the BX-C together pattern the identity of the embryonic segments giving rise to adult structures by activating specific developmental pathways. These genes act alone or in combination to generate specific gene expression profiles associated with particular body segments. Moreover, the activation of a gene at a specified parasegment is maintained posteriorly. Interestingly, the ANT-C and BX-C are spatially organized along the anterior-posterior (A-P) axis of the embryo according to their time of expression, where ANT-C genes are first expressed followed by genes in the BX-C. This phenomenon is referred to as "colinearity."

Similar to *Drosophila* embryonic patterning, the restriction localisation of homeotic gene expression in mammals is necessary for the proper patterning along the A-P and other embryonic axes. The expression of 39 homeobox genes (*Hox*), grouped in four distinct homeotic clusters (*HoxA-HoxD*), determines the identity of cells giving rise to final embryonic structures (Krummlauf, 1994). The mammalian *Hox* genes, like their ANT-C and BX-C counterparts in the

fly, are arranged in 3' to 5' in the clusters corresponding to their sequential time in expression, and more posterior HOX proteins are functionally dominant over anterior ones (Duboule and Morata, 1994; Mann and Morata, 2000). The identification of a specific DNA regulatory element upstream of the HoxD cluster, required for the proper colinear expression of HoxD genes, suggests that a repressive mechanism is installed to prevent more posterior genes from acting prematurely (Kondo and Duboule, 1999). Although the transcription initiation of *Hox* genes occurs at the most posterior region ("*Hox* induction field") of the embryo, gastrulation movements ensure that different cells are continually exposed to specific *Hox* expression profile at the correct time in development (Tam and Beddington, 1987; Lawson et al, 1991). Studies have implicated retinoic acid (RA) and fibroblast growth factors (FGFs) as potential early *Hox* inducers since alteration of either of these signals can modulate *Hox* gene expression (Partanen et al., 1998; Simeone et al., 1990). Moreover, the identification that the *cdx* family, the mammalian homologue of the *Drosophila* maternal *caudal*, controls *Hox* gene expression, reinforces the conservation of *Hox* genes as direct targets by early patterning factors (Subramanian et al., 1995; Charite et al., 1998).

In addition to the selector gene hypothesis, control of cell proliferation rate has been proposed to be an additional mechanism through which *Hox* genes may generate diversity (Dolle et al., 1993; Kostic et al., 1994; Herault et al., 1999). The fact that mammals have budding-out segments during development underscores the impact of cell proliferation. Accordingly, homeotic transformations in *Hox* mutants have not been observed in the limb, which is generated by a mechanism of branching and segmentation and which is not derived from pre-existing modules (Duboule, 1995). The existence of altered blood cell proliferation in *Hox* mutants further substantiates the idea (Bjornsson et al., 2001; Buske et al., 2001). In addition to proliferation defects, anterior/ posterior transformations have been documented among different *Hox* gene mutants along the vertebral column (Rijli et al., 1993).

Although the mechanism(s) by which the *Hox* genes act to generate body segment identity in mammals encompasses additional layers of complexity as compared to flies, the association of two families of proteins, Polycomb group (Pc-G) and trithorax group (trx-G), has been shown to be essential in mammals and in flies for continued *Hox* gene expression beyond the time *Hox* inducers have decayed ("cellular memory"). PcG and trxG use epigenetic features

for their function and elicit additional activities from nucleosome remodelling complexes and DNA/protein modifying enzymes to insure cellular memory.

1.3 Polycomb and Trithorax Protein Families

The PcG protein family was initially discovered by studies of mutants exhibiting posterior transformation of body segments in *Drosophila melanogaster*. Since trxG mutants exhibit anterior transformation of body segments, it was hypothesized that these two sets of protein families possess counteracting functions. Importantly, mammals mutant for PcG or trxG members exhibit corresponding posterior and anterior transformations. On the basis of mutant phenotypes, PcG members are thought to maintain repressed gene expression while trxG maintains activated states (Fig 1.1). With the exception of *pleiohomeotic* (PHO), the *Drosphila* homolog to *YY1*, no other PcG/trxG member was found to bind directly to DNA (Brown et al., 1998). To date PHO has not been reported to bind to any of the other PcG/trxG members. Therefore, it appears that the faithful maintenance of particular gene expressions through mitosis by PcG/trxG involves an unknown mechanism, which tethers appropriate complexes onto specific response elements (Polycomb Response Elements (PREs) or trithorax Response Elements (TREs)) (Cavalli and Paro, 1998). A consensus DNA sequence for PREs/TREs has yet to be identified.

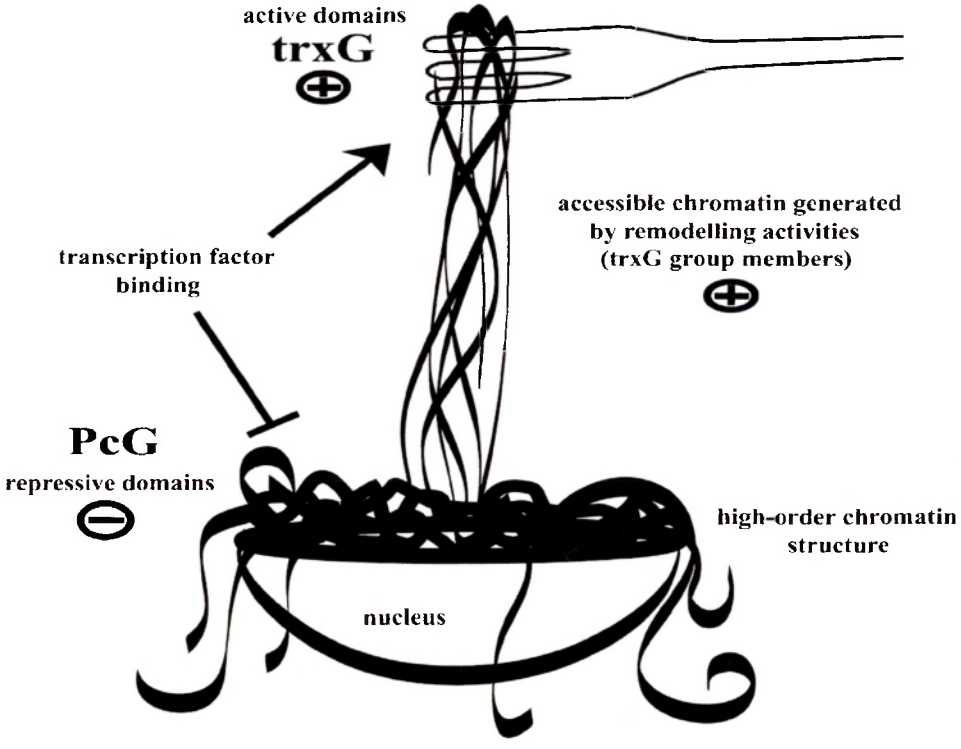


Figure 1.1. A spaghetti model to explain PcG/trxG function. Like spaghetti, particular chromatin domains consist of intermingled strands coiled within one another, thereby creating a mostly inaccessible environment to transcription factors. However, at certain parts, there are strands that are able to escape the repressive force of high-order structure, and consequently, form an activated domain. PcG and trxG members are classically thought to maintain specified gene expression by acting through these high-order chromatin structures. PcG facilitates the formation of heterochromatin-like structures, allowing repression of gene targets, while trxG counteracts this obstruction through its nucleosome remodelling activities.

1.3.1 Insights into PcG mechanism(s)

The PcG family appears to require inactive templates determined by early patterning transcription factors for repression. Two hypotheses have been formulated to account for PcG repression, which can only occur in cells, which lack enhancer activity (Poux et al., 1996; Poux et al., 2001). The first explanation postulates that PcG complexes are stabilised by similar molecular chromatin structures exhibited by the repressed locus, whereas the second considers PcG repression as a default state for PREs. In the latter case, actively transcribed genes would have to prevent the assembly or destabilisation of PcG complexes.

PcG repressed chromatin structure has been explained by the ability of PcG complexes to spread to adjacent genes, generating a repeated array of heterochromatin-like structures (Paro, 1990), or alternatively, by the formation of PcG repressed higherorder chromatin structures over

long distances (Pirotta, 1998). Studies of PcG regulated genes *in vivo* appear to support the second explanation as to how PcG may repress its target genes. Chromatin formaldehyde cross linking immunoprecipitation (X-Chip) experiments revealed that PcG members bind to PREs and to promoter regions, and appear to not spread significantly to adjacent DNA sequences flanking the PREs (Orlando and Paro, 1993; Strutt et al., 1997; Orlando et al., 1998). The dual binding by PcG members at the promoter and at PREs is reminiscent of enhancer-promoter scenarios where protein-protein interactions are required to bring enhancers close to promoters for transcription activation. Analogously, PcG members may ensure inactivity of target genes by joining PREs and promoters via PcG interaction. In support of this idea is the long time observation that PcG repression is dependent on protein dosage and is pairing sensitive, and can compete with transcription factors for DNA binding (Francis and Kingston, 2001; Hodgson et al., 2001; Zink and Paro, 1995). The recent report that general transcription factors (GTFs) are present at PcG repressed loci proposes that PcG may also somehow inihibit GTFs from activating transcription (Breiling et al., 2001). A similar mechanism has been suggested for SIR (Silent Information Regulatory) proteins, which are involved in heterochromatin silencing. Here, evidence has demonstrated that SIR blocks a step downstream of the recruitment of the RNA polymerase II pre-initiation complex, rather than limiting factor accessibility (Sekinger et al., 2001). Furthermore, DNA accessibility is not altered in PcG-repressed loci as would be expected if spreading by PcG complexes indeed coat the DNA fiber (McCall and Bender, 1996; Boivin and Dura, 1998), although a recent study has demonstrated that transcription and site-specific recombination are restricted in *Polycomb*-repressed segments (Fitzgerald and Bender, 2001). In this respect, an interesting consideration is that distinct bindings by PcG at particular sites, as indicated by X-Chip experiments, could in fact represent spreading of PcG complexes along higher order chromatin fibers, which would not be dependent on the DNA sequence (Francis and Kingston, 2001).

The findings that PcG members aggregate at discrete nuclear domains in both the mouse and the fly implicate nuclear domains as an additional mechanism for PcG silencing. The well-established observations of telomere silencing in yeast, of centromeric silencing in mammals, and of limited gene activity by boundary insulators in *Drosophila* suggest that nuclear compartmentalization may be important for gene silencing. The association of different

developmental genes with scaffold associated regions (SARs) for proper development in *Xenopus* underscores the relevancy of nuclear domains in development (Vassetzky et al., 2000). Although not much is known about PcG bodies, the fact that these bodies have been observed in human cell lines and in nuclei of *Drosophila* embryos suggest that nuclear domains are required for proper PcG action. In support of this speculation is the association of the disruption of promyelocytic leukaemia (PML) bodies with acute leukaemia by the PML-RARα fusion (Hodges et al., 1998).

1.3.2 Identification of two classes of PcG complexes

Co-immunoprecipitation data and chromatin immunoprecipitation results of PcG and trxG members have confirmed the significance of protein complex mediated function, since colocalisation of PcG and trxG members at particular chromosomal response elements was observed (Zink and Paro, 1989; Franke et al., 1992; DeCamillis et al., 1992; Rastelli et al., 1993; Chinwalla et al., 1995; Tripoulas et al., 1996). For PcG members, two classes of complexes have been reported. The first appears to play a role in the initial establishment of PcG silencing, while the second may take over the maintenance function once PcG silencing has materialised.

The first class consists of PcG complexes comprised of Enhancer of Zeste (E(z)) and Extra Sex Combs (Esc). The association between E(z) and Esc appears to form the core of this complex (Tie et al., 1998; Jones et al., 1998). In addition, Rpd3 histone deacetylase and p55, a histone-binding protein found in the NURF nucleosome remodelling complex, have been reported to bind to this complex core (Tie et al., 2001). The early Esc expression in development and the transient requirement of Esc during PcG silencing suggested that Esc containing complexes may only function during the early stages of PcG repression (Simon et al., 1995; Struhl and Brower, 1982). Unlike Esc, E(z) is important for additional PcG binding onto chromosomes in later embryonic stages, indicating that E(z) is required for continuous repression of at least some PcG target sites (Rastelli et al., 1993).

Recently, the second class of PcG complex, PRC1 (Polycomb Repressive Complex 1), was purified and was found to consist of PC (Polycomb), PH (Polyhomeotic), PSC (Posterior Sex Combs), and SCM (Sex comb midleg) (Saurin et al., 2001). In addition to PcG proteins,

cytoskeleton components, histone deacetylases, and pol II associated proteins have been reported to stably associate (Saurin et al., 2001). Although not much is known on how PcG complexes repress their target loci, the ability of PRC1 to bind to PREs and to prevent nucleosome remodelling by the SWI/SNF complex implies that PRC1 acts at the nucleosome level for its silencing function (Shao et al., 1999). In support of this, Pc has been reported to bind to core histones (Breiling et al., 1997).

1.3.3 TrxG function depends on chromatin remodelling activities

The trxG family consists of a diversified repertoire of proteins. Members of this family have been reported to remodel nucleosome via ATP dependent activity and to modulate chromatin structure by yet undefined mechanisms. Since trxG members are associated with chromatin, trxG mediated activation of genes appears to occur at the chromatin template.

The best studied nucleosome remodelling complex in *Drosophila* is the brahma (Brm) complex which is analogous to the SWI/SNF family of chromatin-remodelling complexes (Tamkun et al., 1992; Papoulas et al., 1998; Kal et al., 2000). The Brm complex is composed of around ten proteins, including Mor (Moira), Snr1 (Snf5-Related 1), and Osa (Crosby et al., 1999; Dingwall et al., 1995; Collins et al., 1999). Brm, Mor and Snr1 appear to form the core of this complex, while Osa has been speculated to specify target genes for Brm activity (Collins et al., 1999). The Brm complex, like the SWI/SNF remodelling complex in yeast, uses ATP energy to change nucleosome structure, either to permit or to prevent gene activity. In particular, SWI/SNF remodelling includes the ability to slide nucleosomes, to transfer histone octamers to naked DNA, to alter nuclease susceptibility and the topology of closed circular nucleosomal templates (Flaus and Owen-Hughes, 2000; Vignali and Hassan, 2000). In addition to the above complex, Brm has been reported to associate with ASH1 and ASH2 to form to distinct complexes (Papoulas et al., 1998).

The direct association of TRX (Trithorax) protein with Snr1 suggested that recruitment of nuclesome remodelling may be essential for opening of chromatin mediated by TRX (Rosenblatt-Rosen et al., 1998). In addition, the interactions between ASH1 (Absent, Small, Homeotic discs; Tripoulas et al., 1994) and TRX and between ASH1 and histone acetyltransferase, CBP (CREB Binding Protein; Kamei et al., 1996), implies that modification of

the N-terminal sequences of histones also acts to keep chromatin open (Bantiginies et al., 2000; Rozovskaia et al., 1999; Petruk et al., 2001). On the other hand, recruitment of SWI/SNF or Brm activity by Osa to repressive Wingless target genes expands an emerging theme that depending on the chromatin context, trxG members may also suppress gene activity (Collins et al., 1999). In support of this, GAGA, which is encoded by *trithorax-like*, synergises with another remodelling complex, NURF for its activity and has been found to interact *in vitro* with PcG complexes (Tsukiyama et al., 1995). Likewise, Zeste, another trxG member, has been reported to function in both trxG-mediated activation and PcG-mediated repression (Rastelli et al., 1993; Kal et al., 2000). GAGA and Zeste both bind to unique DNA sequences, and have been postulated to recruit both PcG and trxG complexes (Kal et al., 2000; Gildea et al., 2000).

The isolation of TAC1 (Trithorax Acetylation Complex 1) demonstrated that trithorax (TRX) in association with CBP is required for the proper regulation of gene expression of the homeotic gene, *ubx* (ultrabithorax) (Petruk et al., 2001). Like the TRX mammalian counterpart MLL (Mixed Lineage Leukemia), TRX was found to stably interact with Sbf1 (see Section 1.3.6). Sbf1 and CBP appear to be closely linked in the maintenance of *Hox* gene expression, presumably through their interaction with TRX (Petruk et al., 2001).

1.3.4 Dual functions for limited members of the PcG/trxG families

PcG and trxG members are not just responsible for the maintenance of gene repression and activation, respectively, as briefly discussed in Section 1.3.3. Mutants of different members of both families have been found to exhibit both maintenance of activated and repressed gene activity. The best example to date is E(z), which although classified as a Polycomb group member, can actually maintain activated gene expression (LaJeunesse and Shearn, 1996). Recent work by the Shearn laboratory has clearly affirmed the dual function for PcG and trxG proteins. The authors identified several PcG members in a screen for enhancers of a trxG mutant, *ash1* (Gildea et al., 2000). Three conclusions can be extrapolated from the study. The first is that trxG and PcG members interact with one another in the establishment of a functional complex regulating gene expression. Second, although it was previously thought that PcG and trxG have separate binding sites, termed PRE and TRE, respectively, it is clear that certain members from

both groups can bind to the same response element. The documentations that the PRE and TRE within the regulatory element (*bxd*) of ultrabithorax, in the BX-C, partially overlap, as well as the modulation of *Fab-7* (a PRE regulator of Abdominal B gene in the BX-C) mediated repression by both trxG and PcG genes, already hinted to this idea (Tilib et al., 1999; Cavalli and Paro, 1999). Finally, in addition to a PcG and trxG group family, a third family of proteins involved in the maintenance of gene expression appears to exist. This third group, ETP (Enhancer of *trithorax* and *Polycomb*), includes members of PcG and trxG which exhibit the dual property of maintaining both activated and inactivated states (Gildea et al., 2000). In the light of these new results, it has been suggested that elements bound by both PcG and trxG members should be renamed to MEs for Maintenance Elements (Brock and van Lohuizen, 2001).

1.3.5 PcG /trxG conservation in mammals

Like in *Drosophila*, mammalian PcG/trxG members are also responsible for maintaing the correct expression of homeotic and other unidentified target genes. Conservation of PcG/trxG function between *Drosophila* and mammals has been well documented (Table 1.1; Gould, 1997; van Lohuizen, 1999; Brock and van Lohuizen, 2001). In addition to the similar homeotic transformation of trxG/PcG mutants in mammals and in flies, the best examples supporting PcG/trxG conservation include: (i) the demonstration that M33, a mammalian PC homologue, functionally rescues, at least in part, *pc* null mutants, and (ii) that Bmi-1, a mammalian Psc homologue, co-localised with Pc on polytenes, indicating that Bmi-1 can integrate into functional PcG complexes (Müller et al., 1995; Sharp et al., 1997).

Drosophila proteins	Mammalian Homologues
PcG	
Polycomb	M33, MPC2
Polyhomeotic	Mph1/rae28, Mph2
Extra sex combs	EED
dRing	Ring1a, Ring1b
Pleiohomeotic	YY1
Polycomb-like	PHF1
d-Mi2	Mi2
Multi sex combs	---
Super sex combs	---
trxG	
trithorax	MLL
ash1	hASH1
brahma	mBrm, BRG1
Snf5 Related 1 (Snr1)	hSnf5/ ini1
ETP	
Additional sex combs	Asxl1, Asxl2
Enhancer of Polycomb	Epc1, Epc2
Sex combs on midleg	Scml1, Scml2, Scmh1
Posterior sex combs	Bmi-1, Mel18
Enhancer of Zeste	Enx1,Enx2
Suppressor of zeste 2	Bmi-1, Mel18

Table 1.1. PcG/trxG members are conserved between mammals and flies. PcG: Polycomb Group, trxG: trithorax Group, ETP: Enhancer of *trithorax* and *Polycomb*.

Based on the heterogeneous phenotypic patterns in PcG null mutants, two classes within the PcG family can be considered to exist. The first class is termed PcG initiating (PcGi), the members of which first take over the function of early repressors during development. Mutants of these proteins cause very early lethality in mouse development (Eed and Enx1/EZH2). The second class comprises the more classical PcG proteins such as M33, Bmi-1, Mel-18, Ring1B, and so forth, and is called PcG maintenance (PcGm). This set of proteins is proposed to function by stabilising gene expression of early embryogenesis. Mutants of PcGm in general cause subtle morphological transformations, due to slight derepression of *Hox* gene clusters.

Unlike in *Drosophila*, the partial redundancy present in the mammalian system, especially seen for the PcGm proteins, provides an additional layer of complexity in gene regulation. As an example, Bmi-1 and Mel-18 were found to be homologues of the *Drosophila melanogaster* Posterior Sex Combs (Psc). Psc is a member of the PcGm family. Bmi-1 null

mutant mice show only subtle anterior shifts of certain *Hox* gene expression boundaries during development. Mel-18 -/- mice likewise, display similar subtle defects (van der Lugt et al., 1996; Akasaka et al., 1996). The clear indication that Mel-18 and Bmi-1 overlap in function, was provided by double null mutant mice (Mel-18 -/-, Bmi-1 -/-) whose phenotypes are more severe than the single mutants (Akasaka et al., 2001). These mice die at an earlier stage at E9.5 in development and are characterised by severe, aberrant *Hox* gene expression boundaries. In addition to Mel-18 and Bmi-1 null mice, M33, a *Drosophila* Pc homologue, and Eed, a *Drosophila* homologue of Esc, have also been generated. Like the single null mutant mice, M33 null mice exhibit weak posterior skeletal transformation, indicative of a polycomb-like function and of additional mammalian proteins with similar functions (Coré et al., 1997). However, in contrast to most PcGm members, Eed, a PcGi protein, exhibited early embryonic lethality (Schumacher et al., 1996).

Co-immunoprecipitation experiments have shown that like in *Drosophila*, mammalian PcG members are stably associated in protein complexes. Two such different complexes have thus far been identified. One consists of PcGi members including the mouse homologues of *Drosophila* E(z), Enx1 and Enx2, and of *Drosophila* Esc, Eed (van Lohuizen et al., 1998; Seewalt et al., 1998; Ng et al., 2000). As discussed above for the *Drosophila* E(z)/Esc complex, the mammalian counterpart has also been postulated to provide the first recognition of PcG onto its regulatory sites, as exemplified by the report that in *eed* -/- mutants, arrest in development occurs prior to *Hox* gene expression (Simon et al., 1995). The second complex constitutes interactions among members of PcGm, M33, MPc2, Mph1/rae28 and Bmi1 or Mel-18, and is analogous to the *Drosophila* PC, PH and PSC complex (Alkema et al., 1997; Strutt et al., 1997).

Likewise, the activities of trxG members in the regulation of *Hox* genes in mouse have been well conserved both in structure and in function. *Mll* (Mixed Lineage Leukemia) knock-out mice, the mammalian homologue of *Drosophila trithorax*, die at E10.5 showing multiple patterning defects in neural-crest derived structures (Yu et al., 1995; Yu et al., 1998). Heterozygous mice display anterior and posterior homeotic transformations. Similar to mutants of *trithorax*, correct initiation of *Hox* genes expression is observed in *Mll* -/- embryos, but subsequent maintenance of this expression is lost. The reports of trxG misregulation with alterations in cell proliferation have also implicated control of cell cycle as a regulatory target by

certain trxG members. For example, the hBRM and BRG-1, murine homologues of *Drosophila* Brm, were found to bind to "active" retinoblastoma (Rb) protein to induce cell cycle arrest (Dunaief et al., 1994; Zhang et al., 2000). hBRM may recruit the activities of the SWI/SNF remodelling complex to regulate gene activity (Reyes et al., 1997). The reduction of hBRM levels appears to facilitate cell transformation (Muchardt et al., 1998), whereas the human homologue of SNF5 (encoded by the *Ini1* and homologous to *Drosophila Snr1*) has been identified as a *bona fide* tumour suppressor (Versteege et al., 1998; Klochendler-Yeivin et al., 2001). The regulation of cell cycle control is not only limited to trxG members, but also involves the actions of PcG (see Sections 1.3.6 and 1.4.3).

1.3.6 New roles for PcG and trxG members

PcG and trxG members are classically thought to act only at the chromatin fiber of developmental genes, like homeotic clusters, in maintaining proper gene expression. However, several observations linked chromatin proteins with cell cycle regulation. The identification of functional activities by several motifs shared among PcG/ trxG members is potentially insightful in elucidating the mechanisms behind PcG/ trxG actions. The pioneering works are described in detail below.

Studies with Bmi-1, the mouse homologue of Psc, first provided evidence that PcG-mediated regulation directly targets cell cycle control genes. In Bmi-1 overexpressing mice, two tumour suppressor genes within the *ink4a* locus were found to be downregulated, thereby demonstrating that immortalisation of lymphoid cells overexpressing Bmi-1 is possible (Jacobs et al., 1999a; Jacobs et al., 1999b). Additional data with Bmi-1 knock-out mice showed for the first time a mammalian target gene directly regulated by a PcG group member (see Section 1.4.3; Jacobs et al., 1999). Since then, HPC2 (the human homologue to *Drosophila* Pc) in conjunction with Ring1 (the human homologue to *Drosophila* dRing), has been demonstrated to be important for cell cycle arrest by Rb (Dahiya et al., 2001). In addition, Eed has been shown to antagonise Bmi-1 effects on blood cell proliferation, demonstrating that like in *Drosophila*, some PcG/trxG proteins exhibit a dual function in both repressing and activating target genes (Lessard et al., 1999).

A second link with cell cycle regulation has been identified by studies on the SET domain. The SET domain was initially identified as a homology region among three proteins, Su(var)3-9, Enhancer of Zeste (which belong to the PcG family of proteins; Jones et al., 1990), and Trithorax (Tschiersch et al., 1994). For years, the possible role of SET remained elusive, although it was becoming apparent that proteins belonging to both PcG and trxG contained the SET domain. Interestingly, it was shown that SET domains are able to interact with other SET domains as well as with chromatin remodelling proteins (INI1/SNF5) (Rozenblatt-Rosen et al., 1998; Rozvoskaia et al., 2000). A clue to the role of SET in cell cycle regulation was revealed by the finding that the SET domains of mammalian MLL, of *Drosophila* E(z), and of yeast SET1 interact with Sbf1, a member of the dual-specificity phosphatase family (dsPTPases) (Cui et al., 1998). The dual-specificity phosphatase family includes myolotuburin, which is involved in X-linked myotubular myopathy, and Sbf1. The interaction between Sbf1 and the SET domain is interesting, since Sbf1 contains an inactive phosphatase domain. Importantly, it is known that proteins containing a phosphatase domain, whether active or inactive, can bind to a phosphorylated target protein. It was suggested that possibly MLL, when phosphorylated, is bound by Sbf1. This binding may shield MLL from associating with its endogeneous, active dsPTPases, and thus, allows continued proliferation by possibly maintaining the MLL phosphorylated form. Indeed, overproduction of Sbf1 in NIH 3T3 cells causes cell transformation (Cui et al., 1998). These experiments point to the involvement of proper MLL activity in cell cycle regulation.

Furthermore, the SET domain of human Su(var)3-9 (hSUV39H1) was shown to remarkably possess methylating activity of histone H3 (Rea et al., 2000). This activity, however, was not present in all SET domain-containing proteins. Most importantly, MLL was found to lack this particular methylating activity. In the prototypic protein Su(var)3-9 it turned out that in addition to the SET domain, adjacent regions with high cysteine content, were required for the activity. If these cystein-containing regions were mutated or absent (as is the case in other proteins containing the SET domain), the H3-methylating activity was lost. Moreover, the group was able to modulate the activity by introducing marks on histones. In particular, they focused on the phosphorylation of H3 normally found in condensed, heterochromatic chromatin. When phosphorylated histones were offered as a substrate to

Su(var)3-9, methylation of H3 was not detectable. Likewise, in the reciprocal experiment, methylation of H3 histone prevented subsequent phosphorylation. It appears that modification of histones by specific SET domains is tightly regulated to cell cycle dependent modulation, since aberrant mitotic divisions were observed in hSUV39H1 mutant cells. Moreover, the finding that HP1 and H3 methylation are recruited to cell cycle genes by the Rb protein further indicates that this type of mechanism plays an important role in the regulation of cell proliferation (Nielsen et al., 2001).

Since Su(var)3-9 is involved in heterochromatic silencing and thus affects position effect variegation, it was suggested that methylation by Su(var)3-9 provides a code for additional silencing proteins to dock onto heterochromatic sites (Rea et al., 2000). This mechanism has been found to be conserved across species. The *C. elegans* homologue Clr4, like the human and *Drosophila* Su(var)3-9, is able to methylate histone H3. Also, a recent report shows that Swi6, the fission yeast homolog of HP1, can only bind at heterochromatic regions when H3 is methylated, and that the phosphorylation of serine 10 inhibits methylation and consequent binding of Swi6, demonstrating an analogous system as found in human cells (Nakayama et al., 2001). Moreover, deacetylation of H3 Lys14 by Clr3 and the presence of a WD-40 containing protein, rik1, were required prior to methylation of histone H3. In light of these recent demonstrations, there appears to be a layer of regulation, which ensures that correct targets are methylated, and that this layer is regulated by the interaction, either directly or indirectly, of several epigenetic factors.

In addition to the role of the SET domain in cell cycle control, several other domains shared among the proteins in the PcG and trxG families, have emerged to associate with specific functions. These include WD repeats, RING fingers, bromodomain and chromodomain. The RING domain of the RNF2/Ring1B protein has been shown to exhibit E3 ubiquitin ligase activity, implicating proteolytic degradation of histones or unknown targeted proteins as a possible regulatory mechanism (Lee et al., 2001). The bromodomain recognises acetylated modified histones (Dhalluin et al., 1999; Winston et al., 1999; Owen et al., 2000), whereas direct evidence for the function of WD repeats, other than units mediating protein-protein interaction, is lacking.

On the other hand, the chromodomain appears to have diversified in its function *in vivo*. Initially, the chromodomain was found to be shared between two proteins, Polycomb and Heterochromatin Protein 1 (HP1) (Paro and Hogness, 1991). Both proteins are responsible for repression of gene expression. The artificial exchange of the chromodomains between Pc and HP1 revealed that the chromo domain was responsible for targeting Pc or HP1 to its correct target site of action (Platero et al., 1995). Because the chromodomain did not bind a DNA sequence specifically, it was postulated that targeting by the chromodomain occurs through protein-protein interaction. Interestingly, HP1 was found to bind to the H3 tail methylated at Lys 9 via its chromodomain (Lachner et al., 2001, Bannister et al., 2001). Additionally, the interaction between the chromodomain and untranslated *rox* RNA was required for proper dosage compensation of the X chromosome in *Drosophila* (Akhtar et al., 2000). What prevents the chromodomain from randomly interacting with various elements is not known. Probably, the flanking protein sequences endow at least in part chromodomain's specificity.

1.3.7 The histone code

Although modified histones have been known to exist for over three decades, their importance has re-emerged with the recent identification of several trxG/PcG members' ability to modify histones and/or their association with histone-modifying enzymes. The histone code hypothesis has been formulated to incorporate histone modifications as a set of information affecting regulation of gene activity (Strahl and Allis, 2000; Turner, 2000).

The histone code centralises on the assumption that signalling pathways converge on histones (Cheung et al., 2000). The various covalent modifications on the N-terminal tails of histones, such as acetylation, phosphorylation, methylation, ubiquitination, and ADP-ribosylation, provide a signal for different epigenetic regulators to bind and to regulate gene activity. The hypothesis also predicts that many modifications of histone tails can occur within the same nucleosome. An extension to this idea is the "nucleosome code", which postulates that euchromatin and heterochromatin domains are dependent on their convalent nucleosome modifications (Turner, 2000; Jenuwein and Allis, 2001). Depending on the composition of the modified nucleosomes, various epigenetic states can be created that would impinge on the ability

of a cell to proliferate or to differentiate. It should be stressed that modification on histones at various residues, other than the ones described below, have been reported, although functional significance for those modifications is lacking.

Several covalent modifications have been reported and have been correlated with either gene activity or repression, as well as other cellular processes, as depicted in Table 1.2. The fact that single acetylation of histones can be associated with both repressed and active loci enforces the idea that probably the combination of various types of covalent modifications within one nucleosome, reflect a specific signal. In addition, multi-modified nucleosomes are apparently created by a dynamic interplay between various enzymatic complexes responsible for particular histone modifications. To date, the best understood example illustrating these concepts is the coordinated actions of various enzymes, which modulate the tail of histone H3. As described in detail and in connection with the cell cycle above (see Section 1.3.6), phosphorylation at serine 10 of H3 by the Ipl1/aurora kinase inhibits methylation of H3 at lysine 9 by hSUVAR39H1 (Rea et al., 2000). If H3 at serine 10 is phosphorylated, a tendency for acetylation at lysine 14 of H3 by the GCN5 family (Brownell et al., 1996) is increased. The combination of H3 phosphorylation at serine 10 and acetylation of lysine 14 of H3 creates an epigenetic tag for transcription (Kuo et al., 1996; Lo et al., 2000; Clayton et al., 2000). This tag is also influenced by acetylation of H3 at lysine 9, since a peptide containing acetylated lysine 9, can still be phosphorylated at serine 10 (Krebs et al., 1999). In *Drosophila*, a similar scenario has been reported where phosphorylation of H3 at serine 10 in conjunction with acetylation of H4 at lysine 16 allows enhanced transcription activity from the male X-chromosome (Jin et al., 1999; Turner et al., 1992).

Interestingly, the same phosphorylation tag at H3 serine 10 has been a marker for condensed chromosomes during mitosis (Bradbury, 1992; Koshland and Strunnikov, 1996), and has been linked with the gene expression of *c-jun*, *c-fos*, and *c-myc* (Mahadevan et al., 1991; Chadee et al., 1999). The recent characterisation of an additional phosphorylation site of H3 at serine 28 preferentially associated with mitotic chromosomes suggests that co-phosphorylation of H3 at serine 10 and 28 marks condensed chromosomes (Goto et al., 1999). Analogously, H1 has been reported to be phosphorylated in mitotic chromosomes and its phosphorylation has been linked to transcription (Bradbury, 1992; Koshland and Strunnikov, 1996; Dou and

Gorovsky, 2000). Other cellular processes, such as apoptosis and DNA damage, have been associated with H2B and H2A phosphorylation, respectively (Rogakou et al., 1999; Ajiro, 2000).

In addition to phosphorylation and H3 acetylation, H4 acetylation has been widely documented at lysines 5, 8, 12, and 16. Already mentioned above, acetylation of lysine 16 is correlated with hypertranscribed *Drosophila* male X-chromosome. Acetylation of H4 lysine 5 and lysine 12 appears to allow gene transcription, since mutants which affect silencing exhibit higher levels of acetylation at both loci (Rundlett et al., 1998). However, acetylation of only lysine 12 of H4 is associated with heterochromatin formation in the mating type locus in yeast (Braunstein et al., 1996; Turner, 2000). Although both H3 and H4 are pre-acetylated when incorporated into newly synthesised DNA, only H4 acetylation at lysine 5 and lysine 12 seems to be conserved (Sobel et al., 1995; Kuo et al., 1996). However, some organisms display H3 acetylation at lysine 9 (Sobel et al., 1995). At least for the di-acetylated H4, erasure by deacetylases occurs several minutes after the completion of DNA replication (Taddei et al., 1999).

Finally, the recent identification of methylated histones has spurred several studies directed at understanding the significance of the modification. Methylation of lysine 9 of H3 is correlated with silencing as described in Section 1.3.6 (Nielsen et al., 2001). However, methylation of lysine 4 of H3 and of arginine 3 of H4 have both been reported to allow transcriptional activity, although methylation of lysine 4 of H3 has also been required for repression (Strahl et al., 1999; Litt et al., 2001; Noma et al., 2001; Wang et al., 2001; Boggs et al., 2002; Briggs et al., 2001). As in the case for phosphorylation at serine 10 of H3, methylation of arginine 3 of H4 facilitates subsequent acetylation of H4 at lysines 8 and 12 (Wang et al., 2001).

Modification	Functional Association
H4 (non-Ac)	heterochromatin
H4: K12Ac	silencing, heterochromatin
H4: K5Ac, K12Ac	transcription, chromatin assembly
H4: K8Ac	transcription
H4: K16Ac	transcription
H4: K20Me	transcription
H4: R3Me	transcription
H3 (non-Ac)	heterochromatin
H3: K14Ac	transcription
H3: K9Ac, K14Ac	transcription, chromatin assembly
H3: K14Ac, K23Ac	chromatin assembly
H3: K9Me	silencing, heterochromatin
H3: K4Me	transcription/repression (?)
H3: K4Me, K14Ac	transcription
H3: S10P, S28P	mitotic chromosome condensation
H3: S10P, K14Ac	transcription
H3: S10P, H4: K16Ac	transcription
H3: S10P, H1: P	transcription/silencing (?)
H1: Ubi	transcription
H2A.X: 139P	DNA repair, apoptosis
H2B: 32P	apoptosis

Table 1.2. The Histone Code. Combinations of various histone modifications are speculated to represent different signals affecting gene activity and other cellular processes, as indicated. Ac: acetylation, P: phophorylation, Me: methylation, Ubi: ubiquitination.

1.4 The influence epigenetics has in cancer

1.4.1 Interfering with enzymatic complexes

Characterisation of many complexes possessing acetyltransferase or deacetylase activity has indicated that these activities are essential for proper regulation of genes. It is clear that such

enzymatic activity is necessary for the integrity of the cell (Ikura et al, 2000; Tsai et al., 2000). Although in the majority of the cases, histones were reported to be the target substrates, several other substrates have been identified. Most notably, one report showed that deacetylation of p53 is an important means of regulating p53 activity (Luo et al., 2000). Furthermore, the acetylation of HMGI/Y (high mobility group I/Y) an architectural protein, by PCAF (p300/CBP associated factor; Yang et al., 1996) /GCN5 (Brownwell et al., 1996) acetyltransferase prevented its degradation and allowed enhancement of interferon-β gene expression (Munshi et al., 2001). The CARM1 (coactivator associated arginine methyltransferase 1; Chen et al., 1999) dependent methlylation of the acetyltransferase p300/CBP allows full activation of nuclear receptor target genes while attenuating p300/CBP mediated cAMP-induced transcription, thereby modulating several regulatory pathways (Xu et al., 2001).

Several studies collectively support an emerging theme of interference with proper recruitment or with association of deacetylases by oncoproteins. Recruitment of deacetylases was found to be influenced by oncogenic proteins. It was shown that activation of oncogenic Ras results in an increased expression of HDAC4. Interestingly, this increased expression of HDAC4 was found to be associated with an elevated level of kinase activity. Possibly, the kinase activity associated with the HDAC4 complex transduces signals from the RAS-MAPK pathway. If indeed this hypothesis turns out to be true, Ras mediated cell deregulation might directly act through chromatin remodelling (Zhou et al., 2000). Recruitment of deacetylases by ETO (Eight Twenty One) in acute myeloid leukaemia is crucial for its ability to promote leukemogenesis (Gelmetti et al., 1998; Stunnenberg et al., 1999). The retinoblastoma (Rb) protein, a nodal point in regulating gene repression during the S phase of the cell cycle, also recruits the HDAC1 deacetylase. The presence of an oncoprotein, E7, prevents this association and thus allows activation of target genes (Brehm et al., 1998). Most importantly, oncoproteins such as Set/TAFIB and MTA1 have been identified as members of the INHAT, inhibitor of acetyltransferases (p300/CBP and PCAF), and of the NURD deacetylase and remodelling complexes, respectively (Seo et al., 2001; Xue et al., 1998). The recent report showing that BRCA1 transcription is repressed by histone hypoacetylation and chromatin condensation, and consequently, the predisposition of breast epithelial cells for cancer by BRCA1 misregulation, perfectly illustrates how an epigenetic misregulation can be a first step in tumourigenesis (Rice et

al., 2000). In addition, the interaction between the RING finger of tumour-suppressor BRCA1 and its associated partner BARD1, which harbours an E3 ligase activity that is inactivated in breast cancer-derived mutations, again shows that enzymatic activity is a target in tumourigenesis (Hashizume et al., 2001; Brzovic et al., 2001).

1.4.2 Nucleosome remodelling connecting with cancer

A flurry of reports has demonstrated interactions between nucleosome remodelling complexes and cell cycle regulators (Harbour and Dean, 2000). Chromatin remodelling and transcription factor accessibility are both required for gene activation. The improper recruitment of such complexes to loci that should be kept repressed can easily result in deregulation of cell function. Therefore, reports of INI1/SNF5 deletion at chromosome 22, which correlates with malignant rhabdoid tumours and lymphoid malignancy, are not surprising (DeCristofaro et al., 1999: Yuge et al., 2000). Moreover, mutations in BRG1, also a member of the human SWI/SNF remodelling complex, have been associated with multiple human tumour lines, including breast cancer. The direct association of BRCA1 with BRG1 has recently been shown (Bochar et al., 2000). The presence of a dominant-negative mutant of BRG1, or of a cancer-causing deletion mutant of BRCA1, abrogates transcription activation of BRCA-1 upon p53 signalling. BRG1's interaction with Rb, which is required to repress cyclin E and A and cdc2, provides an alternative mode of action which when abrogated can lead to tumourigenesis (Zhang et al., 2000).

1.4.3 Bmi-1 in cancer

The activities of PcG and trxG in cell integrity and cell proliferation were underscored by the finding that members of both groups are often mutated in several types of cancer (also described above). The best studied PcG/trxG proteins which are implicated in cancer and cell cycle control are Bmi-1 and MLL. Bmi-1 was found to be a potent collaborator of the *c-myc* oncogene in lymphomogenesis (van Lohuizen et al., 1991a; van Lohuizen et al., 1991b), while *Mll* is consistently translocated and fused with several partner genes in lymphoid and myeloid derived leukaemia (see Section 1.5; Ayton and Cleary, 2001).

The synergistic actions between Bmi-1 and c-Myc were explained by the recent connection of Bmi-1 as a regulator of an important cell cycle control gene, the ink4a-Arf locus (Jacobs et al., 1999). The mechanism(s) through which Bmi-1 acts remains unknown. Apparently, cell transformation was achieved by the repression of tumour suppressor genes present in the ink4a-Arf locus, through the over-expression of Bmi-1, and by subsequent deregulation of cell cycle progression by c-Myc. The Bmi-1 and ink4a-Arf connection was confirmed to be relevant since cells lacking Bmi-1 expression undergo premature senescence. This is due to the derepression of the ink4a-Arf locus, resulting in the expression of $p16^{ink4a}$ and $p19^{Arf}$ tumour suppressor proteins. The inactivation of the ink4a-Arf locus rescued this premature-senescent effect and demonstrated that the gene activity status of ink4a-Arf locus is critical for cell immortalisation and senescence. The relevance of this discovery was illustrated by the finding that the same mechanism of repression of ink4a-Arf by over-expressed Bmi-1 was documented in primary human osteosarcoma, in non-small cell lung cancer, and in several forms of non-Hodgkin lymphomas such as Mantle cell B lymphomas (Vonlanthen et al., 2001; Bea et al., 2001; van Kemenade et al., 2001). These observations indicate that overexpression of Bmi-1 or particular PcG proteins may contribute to tumourigenesis of lesions where the activity of the ink4a-Arf locus is retained. Additionally, the principle that both cell defence and proliferation control must be mutated for tumourigenesis is reinforced (see Section 1.1).

Mechanistically, the ink4a-Arf products p16 and p19Arf act as upstream regulators of the Rb and p53 tumour-supressor pathways, which act at specific check-points of cell cycle progression (Sherr, 2001). Recent studies strongly argue that the proper regulation of ink4a-Arf is crucial for normal cell integrity, and propose that negative regulators of cell cycle control genes, such as ink4a-Arf, may in fact be unidentified oncogenes contributing to tumourigenesis (Sherr, 2001; Kimperforst et al., 2001; Sharpless et al., 2001). In addition to Bmi-1 itself, the ability to immortalise senescence-predisposed cells lacking Bmi-1 by the over-expression of the T-box transcription factor TBX2 provides strong support for this hypothesis (Jacobs et al., 2000). In this study, the alleviation of cell senescence was mediated by the repression of the $p19^{Arf}$ expression.

1.5 Mixed Lineage Leukemia

1.5.1 The disease

Acute leukaemia is generally characterised by genetic translocations and inversions, leading to the activation of proto-oncogene proteins (Rabbits, 1994; Sawyers, 1997). Acute leukaemia can be derived from either the myeloid (Acute Myeloid Leukemia, AML) or lymphoblastic (Acute Lymphoblastic Leukaemia, ALL) lineages. Some activated proto-oncogenes can lead to the development of both cell lineage leukaemias, such as MLL (Mixed Lineage Leukaemia), the human homologue of the *Drosophila* Trithorax.

As described in detail below, the *Mll* gene, residing at the 11q23 position, is translocated to a variety of partner genes to create an in-frame expressible fusion protein. Activation of this fusion protein has been correlated with the onset of acute leukaemia. It was observed that a high percentage of patients who took a therapeutic drug that inhibits the action of topoisomerase II, developed acute leukaemia and exhibited a break point at the 11q23 locus. This observation underscored the importance of the translocation event at the *Mll* locus, which consisted of several Alu sequences, in generating acute leukaemia. Translocations involving the MLL gene account for up to 80% in leukaemia in infants, representing 23% of cancer diagnoses in children under the age of 15 (Heerema et al., 1994; Pui et al., 1995; Pui, 2000).

The MLL disease is associated with very poor prognosis, with 75-80% of diagnostic cases surviving up to 5 years (Pui et al., 1995; Pui, 2000). In addition to elevated numbers of white blood cells, the disease is also associated with leukaemia of the central nervous system (CNS) (Pui et al., 1995; Pui, 2000). Patients with mutated MLL lesions do not respond well to anti-cancer drugs and chemotherapy, although acute leukaemia derived from activation of proto-oncogenes other than MLL are associated with long-term event-free survival rate of 80% in children (Pui et al., 1994; Saha et al., 1998; Pui, 2000). Gene expression analyses of MLL derived leukaemic samples revealed a highly distinct gene expression profile in comparison to conventional ALL and AML (Armstrong et al., 2002). In fact, these differential gene expression profiles can be used to distinguish MLL-based leukaemic patients from conventional ALL/AML

leukaemic patients with 95% accuracy. This study has proposed that MLL should be treated as a separate disease, and that the distinct pattern of gene activity by MLL associated leukaemia provides an explanation as to why patients with MLL respond poorly to conventional therapies.

1.5.2 Structure homology to TRX

TRX and MLL share two homologous domains at the C-terminus, and one domain at the N-terminus (see Chapter 2: Figure 2.1). At the N-terminus, TRX and MLL both contain two stretches of SNL (Speckled Nuclear Localization), which mediate speckled sub-nuclear localization (Yano et al., 1997). It was conjectured that the disruption of the localisation of both MLL and TRX at nuclear domains may play a significant role for the appearance of leukemia (Yano et al., 1997; Ayton and Cleary, 2001). With the exception of the putative nuclear receptor type DNA binding domain at the N-terminus of TRX (Stassen et al., 1995), the N-terminus of MLL consists of several additional functional sequences. The N-terminal sequence of MLL comprises three AT hooks (Tkachuk et al., 1992), a cysteine-rich region similar to DNA methlytransferase (MTase) (Domer et al., 1993; Cross et al., 1997; Hendrich and Bird, 1998), a repression domain (Zeleznik-Le et al., 1994; Prasad et al., 1995), and an activation domain at its C-terminus (Zeleznik-Le et al., 1994). AT hooks are speculated to bind at minor grooves of DNA via their RGR motif, and are thought to establish open chromatin configuration at AT-rich regions (Reeves and Nissen, 1990; Huth et al., 1997). Interestingly, the third AT hook of MLL shares similarity with an ATP binding site found in HMGI/Y proteins (Zeleznik-Le et al., 1994). HMG proteins are postulated to be involved in maintaining higher order open chromatin structure by possibly competing with histone H1 (Reeves 1992; Huth et al., 1997). Actions at higher order chromatin structure by AT hooks, have been reinforced by the finding that this region binds SARs (Scaffold Attachment Regions), which organise interphase and mitotic chromatin into loops (Gasser et al., 1989; Zhao et al., 1993; Khadake and Rao, 1997). The recent structural resolution of HMGI/Y bound to DNA suggests that MLL binds with weaker affinity to the minor groove, and thus, possibly the weak interaction by MLL may be used to indirectly stablise protein-DNA interactions (Huth et al, 1997). Interestingly, the possibility of AT hooks

phosphorylation links MLL again with regulation by cell cycle dependent phosphorylation (Nissen et al., 1991; Reeves et al., 1991; Ayton and Cleary, 2000). The MTase-like region, as the name implies, consists of a cysteine rich motif (CxxC), which shows homology to DNA methyltransferases (DMT) and methyl binding domain protein 1 (MBD1) (Domer et al., 1993; Cross et al., 1997; Hendrich and Bird, 1998). DMTs are able to methylate DNA, whereas MBDs bind methylated sequences. Shown recently is the ability of the MT domain to bind unmethylated CpG sequences (Birke et al., 2002).

Finally, two important domains in TRX and in MLL, the PHD fingers and SET domain, require special notice since they are not only present in several members of both trxG and PcG families, but have also been thought to play an important role in TRX/MLL mediated gene activity. Although the exact role of the PHD fingers is still not known, they are classically regarded as a moiety that is able to bind DNA. However, PHD fingers in MLL and TRX share greater homology to plant PHD fingers, which are thought to mediate protein-protein interaction (Aasland et al., 1995). The importance of PHD fingers for *Hox* gene regulation was shown by the interaction of the third PHD finger of MLL with Cyp33, a nuclear cyclophilin containing a RNA binding domain known to regulate gene expression by interacting with transcription factors and deacetylase complexes (Fair et al., 2001). In this study, it was shown that Cyp33 interaction with MLL represses HOX expression in tissue culture. Notably, the third PHD finger of MLL is absent in all leukaemic fusion proteins. Therefore, the lack of the interaction with Cyp33 by the fusion proteins could imply that the chimeric proteins are constitutively active. Indeed, overexpression of Cyp33 in leukemic cell lines lacking the third PHD finger, was not found to have any effect on gene expression (Fair et al., 2001).

Several research groups have begun to elucidate in detail the role of the SET domain (see Section 1.3.6). The recent discovery that the SET domain of Su-(var)3-9 possesses a histone methylating activity has encouraged studies to understand why in contrast, the SET domain of TRX lacks the methylating activity. An understanding of this phenomenon was provided by the finding that the SET domain of TRX acts as a histone recognition module, that is essential for TRX regulation of gene expression (Katsani et al., 2001). The z11 *trx* mutant allele which lacks an intact SET domain is pupal lethal and was shown to be incapable of binding to core histones and to nucleosomes (Katsani et al., 2001).

1.5.3 MLL fusion proteins

MLL was initially discovered in leukaemic patients, who consistently possessed translocations between the MLL locus and various fusion partners (Table 1.3; Gu et al., 1992, Tkachuk et al., 1992; Nakamura et al., 1993; Shilatifard et al., 1996). The MLL fusion proteins are translocated at a region called the "Break Point Cluster" which spans parts of the PHD finger of MLL. Notably, the SET domain and at least half of the PHD finger of the full-length MLL are lost and replaced by new C-terminal sequences upon translocation. Since at least 30 different fusion proteins, which contain the N-terminal part of MLL are known to date, this portion was speculated to be of great importance in the predisposition of lymphoid and myeloid derived cell lineages to cancer. Additional functions contributed by the C-terminal fusion partner of the chimeric proteins were postulated to enhance loss of cell proliferation control. Clear evidence supporting the model is lacking.

Among the characterised MLL fusion proteins, two classes can be formulated based on the latency period required for the onset of leukaemia (Ayton and Cleary, 2001). MLL-ELL, MLL-CBP, MLL-βgal, and MLL-PTD display AML after a long gestation period, whereas MLL-AF4, MLL-AF9, MLL-ENL, and MLL-AF10 are associated with aggressive AML and ALL. Accordingly, MLL-CBP, MLL-ELL, and MLL-PTD are generally associated with elderly patients whilst the more aggressive translocations, particularly MLL-AF4 and MLL-AF9, are correlated with infants aged a few weeks old to a few years.

Gene	Localization	Domains in Fusion Protein		Disease
		Retained	Lost	
MLL	11q23	SNL1&2, AT Hooks	SET, PHD	ALL & AML
AF1q	1q21	whole gene product	?	AML
AF4	4q21	transactivation, CHD	?	ALL
AF9	9p22	transactivation, NLS	?	AML/ALL
AF6	6q27	kinesin, myosin	Ras activation	AML/ALL
AF10	10p12	LZ, Q-rich	PHD	AML
AF17	17q21	LZ, Q-rich	PHD	AML
AFX	Xq13	?	DNA binding	AML
ELL	19p13.1	R2, R3, R4	R1	AML
ENL	19p13.3	transactivation, NLS	?	AML/ALL
CBP	16p13	BR, HAT	PHD, CREB(?)	AML
p300	22q11.2	BR, PHD, Q-rich	CREB, ZF	AML

Table 1.3. MLL Partners. The 12 most common MLL partners are depicted above. The name, localisation, and domains retained or associated with the fusion protein, are described. In addition, the leukaemic disease, whether derived from the myeloid (AML) or lymphoid (ALL) lineages, is given. SNL: speckled nuclear localisation, CHD: C-terminal homology domain, NLS: nuclear localisation signal, LZ: leucine zipper, Q-rich: glutamine rich, BR: bromodomain, HAT: acetyltransferase, ZF: zinc finger, R1-R4: domains in ELL.

The C-terminal partners of MLL leukaemic fusion proteins do not appear to share any common functions, although many of these MLL partner genes are generally ubiquitously expressed. ELL was isolated biochemically by its ability to act as a transcriptional elongation factor for RNA polymerase II (Shilatifard et al., 1996). Its central R2 domain, which is present in the fusion protein, prevents pol II pausing along the DNA template, thereby increasing the rate of RNA synthesis (Shilatifard et al., 1997). Interestingly, this R2 domain was found to be dispensible for myeloid immortalisation by MLL-ELL, although another conserved region of ELL, R4 was required (DiMartino et al., 2000). The interaction of R4 with EAF1 (ELL associated factor 1; Luo et al., 2001), which intriguingly is homologous to AF4, another fusion partner of MLL, facilitated transactivation by ELL's R4 (Lavau et al., 2000).

ENL, like ELL, possesses transactivation domains which were found to be conserved with ANCl (actin non-complementing), a component of the SWI/SNF chromatin remodelling complex in yeast and which is thought to endow dominant gain of MLL function by keeping MLL target genes active (Cairns et al., 1996; Saha et al., 1998; Rowley, 1998; Ayton and Cleary,

2001). Both the AT hooks and CxxC motif of MLL and the last 84 amino acids of ENL domains were required for myeloid transformation (Slany et al., 1998). In addition, another partner gene, AF9 shares extensive homology with ENL (Nakamura et al., 1993). Both acquire a NLS signal and transcription transactivation domain (characterized by serine and proline rich stretches), which has been shown to be important for their ability to transform cells (Slany et al., 1998). Although the function(s) for AF9 in normal cell progression is not known, it has been speculated that many fusion partners (such as AF9, AF4, ELL, and ENL) appear to interact with the RNA polymerase II transcription machinery (Prasad et al., 1995; Rubnitz et al., 1994).

Like the above fusion partners, AF10 also uses transcriptional effector properties to derail the rate of cell proliferation. The LZ domain of MLL-AF10 in conjunction with a conserved octapeptide sequence (EQLLERQW) was sufficient for myeloid immortalization and was able to activate *Hoxa7*, a MLL target gene (Ayton and Cleary, 2001). Although transactivation potential by MLL-AF10 is weaker than for MLL-ENL or MLL-ELL, transplantation of MLL-AF10 into immuno-compromised mice resulted in aggressive AML, developing within 60 days (Ayton and Cleary, 2001).

The AF4 gene, when translocated to MLL, is associated predominantly with infant pro-B ALL (Domer et al., 1993; Nakamura et al., 1993; Johansson et al., 1998). Supporting a unifying theme, AF4 acts as a transcriptional activator when fused with the GAL4 DNA binding domain (Prasad et al., 1995). The central conserved domain was found to mediate this potent transcriptional activation (Ma and Staudt, 1996; Morrissey et al., 1997). In addition to AF4, other family members, such as AF5q32 and LAF4 have been reported to fuse with MLL (Taki et al., 1999; von Bergh et al., 2001). The generation of null AF4 mice revealed that AF4 is required for proper hematopoietic development, particularly in the transition between pre-B cell to mature B cells (Isnard et al., 2000). The identification of the *Drosophila* AF4 homolog, *lilliputian*, connected MAP kinase (MAPK) and PI3-kinase/ Protein Kinase B (PI3K/PKB) pathways as possible additional venues through which the AF4 protein may act (Tang et al., 2001; Wittwer et al., 2001).

Since the identification of CBP as one of MLL's fusion partners, aberrant enzymatic activity has been proposed to be a contributing factor in leukemogenesis. CBP regulates several signalling pathways and was shown to exhibit histone acetyltransferase activity, therefore

implicating CBP as a regulator of transcription at the level of epigenetics. Both the bromodomain and the HAT activity were sufficient to immortalise myeloid cells and allow development of AML *in vivo* (Lavau et al., 2000). The ability of bromodomains to recognise and to bind to acetylated H3 and H4 may tether MLL-CBP to critical target genes and in conjunction with CBP's HAT activity, allow accessibility to the transcription machinery (Dhalluin et al., 1999).

Finally, the surprising report that MLL-βgal can induce AML, albeit with long latency, underscores the importance of the disruption of MLL normal function (Dobson et al., 2000). The fact that duplication of part of the amino-terminal of MLL (PTD) and the absence of MLL exon 8, which contains part of the first PHD finger, are associated with long latency leukaemia, indicate that abrogation of normal MLL function predispose cells for tumorigenesis (Schichmann et al., 1994; Caligiuri et al., 1996; Lochner et al., 1996). How these MLL lesions contribute to the inhibition of normal MLL function is not known.

1.5.4 MLL fusion proteins and apoptosis

In addition to their effect on transcription, MLL fusion proteins have also been implicated in inducing apoptosis (Ayton and Cleary, 2001).

The presence of MLL-AF9 in cells resulted in cell death within 72 hours, caused G1 arrest and cell differentiation, and induced upregulation of p53 target genes (Caslini et al., 2000). However, if the protein concentration of MLL-AF9 is reduced to levels only detectable by RT-PCR, for example, by expressing with a weak endogenous promoter, cells are able to survive, as exemplified by the MLL-AF9 knock-in mice (Corral et al., 1996; Dobson et al., 1999). Both results imply that high amounts of MLL fusion proteins are toxic to the cell, or alternatively, that mis-expression of the protein in cells that do not inherently express appropriate target genes, are susceptible to an apoptotic response (Ayton and Cleary, 2001).

However, the relation between MLL fusion proteins and apoptosis is not absolutely clear since several studies have reported both induction and inhibition of apoptosis directly by MLL fusion protein. The finding that the leukaemic MLL fusion proteins (such as MLL-ENL, MLL-AF9, and MLL-ELL) inhibit GADD34-induced apoptosis whereas wild-type MLL does not, indicates that possibly MLL fusion proteins directly prevent normal cellular apoptotic

response (Adler et al., 1999). Furthermore, the treatment of cells with antisense oligo-nucleotides against MLL-LTG19 or against MLL-AF9 arrests cell growth and induces apoptosis (Akao et al., 1998; Kawagoe et al., 2001). However, it was noted that MLL-ENL and MLL-ELL also arrest cell proliferation by causing cell maturation or by activating apoptosis, respectively (Schreiner et al., 2001; Johnstone et al., 2001). Clearly, the effect by MLL fusion proteins on apoptosis is ambiguous. Nevertheless, in order to generate leukaemia, aversion of cell death by MLL fusion proteins must occur. Whether MLL fusion proteins deactivate the apoptotic response, directly or indirectly remains to be conclusively shown.

1.5.5 Mechanism(s) by which MLL fusion proteins generate leukaemia

A central question in understanding how MLL fusion proteins promote leukaemia is to explain why particular MLL fusion proteins are associated with myeloid (AML) or lymphoid (ALL) derived leukaemia. Two hypotheses have formulated to account for this discrepancy. The first postulates that translocation events between MLL and fusion partners are restricted to cell lineage, and thus dependent on factors that allow illegitimate recombination to occur. The second takes into account that lineage specific leukaemia is largely determined by the MLL fusion partner. The facts that many MLL fusion partners are ubiquitously expressed and that MLL fusion proteins are associated with both types of leukaemia, would indicate that lineage specific transformation by the MLL fusion partner is not absolute.

In vitro cell culture assays and *in vivo* mouse models collectively support the role for MLL fusion proteins as gain-of-function mutations. Whether the disruption of normal MLL function or the additional, potential activities by the C-terminal partners is most crucial for cell transformation is unclear. However, it appears that both events are critical for leukemogenesis.

The importance of the C-terminal partner in the control of cell proliferation was shown in knock-in mice expressing the MLL-AF9 chimeric fusion protein (Corral et al., 1996). It was shown that MLL-AF9 mice exhibited elevated levels of lymphoid cells after several months, while mice expressing the truncated N-terminus of MLL tagged with myc (MLL-myc) remained normal. In addition, MLL-AF9 *in vivo* was found to rapidly increase myeloid cell proliferation,

suggesting that this increase in cell growth rate may facilitate additional mutations for development of leukaemia (Dobson et al., 1999).

The effect on leukemogenesis by the lack of a functional C-terminal portion of MLL (such as in MLL-myc mice) was addressed by introducing the fusion protein, MLL-βgal (Dobson et al., 2000). β-gal has been extensively used as a marker in model systems, is capable of oligomerisation, and has no known tumourigenic effect. Intriguingly, mice expressing the MLL-β-gal fusion developed leukaemia, albeit a milder form than the MLL-AF9 control mice. This study suggested that the partial loss-of-function of MLL and the ability to oligomerise via the β-gal domains, perhaps to stabilise the fusion protein, are sufficient to render MLL-β-gal oncogenic. Self-oligomerisation of the fusion proteins may also represent a gain-of-function necessary for oncogenesis. In support of this proposal, the C-terminal domains of known fusion partners of MLL (for example, AF10 and AF17) contain leucine zippers. However, MLL is also known to translocate to non-oligomerising proteins (such as p300/CBP and ELL), where the fusion proteins cause cancer. In these cases, it could very well be that an additional enzymatic or transcriptional activity contributes to their oncogenicity. Thus, it appears that the C-terminal portion of the fusion protein enhances the cancer phenotype. Furthermore, the ability of MLL fusion proteins to transform myeloid progenitors in a wild-type background (i.e. in the presence of two MLL copies) underscores the importance of MLL C-terminal partners.

An additional consideration to keep in mind is the effect the disruption of the wild-type function of the C-terminal fusion partner has on cell homeostasis. Analogous to MLL, inhibition of the physiological function of the C-terminal partner by the MLL fusion protein may also contribute to leukemogenesis. As an example, the MLL partner, AF6 gene, has been shown to directly transactivate the Fas ligand gene and the tumour suppressor $p27^{kip1}$ to promote apoptosis and cell cycle arrest (Brunet et al., 1999; Medema et al., 2000). Conceivably, the MLL-AF6 fusion protein may interfere with the normal ability of AF6 to induce apoptosis, and thus enhance cell survival to promote leukemogenesis (Ayton and Cleary, 2001). Ultimately, the identification of MLL regulated target genes and the molecular characterisation of the C-terminal partners will be required to unravel how the chimeric fusion proteins modulate the activities of MLL and its C-terminal partners to misregulate cell proliferation.

1.6 Aims of the Thesis

The aim of the work described in this thesis was to develop *Drosophila melanogaster* as a supplementary model system that would provide a deeper understanding of how MLL fusion proteins contribute to the development of leukaemia. Two MLL fusion proteins were selected for these studies. They include MLL-AF4 and MLL-AF9, which account for 40% and 27% of infant acute leukaemia cases, respectively (Ayton and Cleary, 2001).

The thesis addresses several points. First, the establishment of *Drosophila* as a system to study MLL fusion proteins should provide a more feasible means of identifying pathways through which MLL-AF9 and MLL-AF4 misregulate. This system would define the initial contours of the possible physiological functions for AF9 and AF4, which to date, remain unknown. Analyses of the expression of MLL and/or MLL fusion proteins in flies would assert (i) whether the MLL fusion proteins are the sole cause of leukaemia, and (ii) whether chromatin architecture has been compromised. Second, the expression of wild-type MLL as well as of MLL fusion proteins would comment on the relevancy of the C-terminal fusion partner in leukemogenesis. If MLL-AF4 and MLL-AF9 manifest similar molecular phenotypes, a conclusion that disruption of wild-type *Mll*, or its equivalent homologue in the fly, *trithorax*, is sufficient in part to facilitate cell transformation, would be heavily substantiated. Alternatively, different effects by MLL-AF4 and MLL-AF9 would suggest that the AF4 or AF9 portion of the fusion proteins targets specific pathways for deregulation of cell integrity. Third, MLL fusion protein effect on apoptosis could be clarified. The modulation of MLL fusion protein expression levels is simplified with the UAS-Gal4 system in *Drosophila*, and the generation of cells, which either express or do not express the MLL fusion proteins within the same developmental setting, can be assessed for direct apoptotic activity. Finally, the possibility that *trithorax* possesses additional functions required for the completion of *Drosophila* development, as implied by the multitude of *trithtorax* mutants exhibiting diverse phenotypes, can be investigated. Importantly, MLL fusion proteins may target this pathway(s) to account in part for its oncogenicity.

CHAPTER 2

MLL fusion proteins specifically induce lethality at late *Drosophila* development.

2.1 Introduction

The disease Mixed Lineage Leukaemia refers to the over-proliferation of specific blood cells derived from either the lymphoblastic or the myeloid lineages. The 11q23 locus was observed to be consistently translocated and fused to several partner genes throughout the genome in patients suffering from the disease. It was hypothesized that the gene disrupted in 11q23 may have a crucial role in the progression of leukaemia. Subsequent cloning of the locus identified a gene (named *Mixed lineage leukemia, Mll*) that only retained its N-terminus in patients with a translocation involving the 11q23 locus. The discovery that MLL showed homology to the *Drosophila* Trithorax (TRX) protein was a major stepping stone, since the similarity between the two proteins provided a first clue of what the function of MLL may be. TRX is the prototype member of the trithorax group (trxG) family. Together with the Polycomb group (PcG), trxG members are thought to regulate gene expression by maintaining correct higher order chromatin structure associated with either active or inactive genes, including the homeotic gene clusters (*Hox*), set by transcription factors during early embryogenesis.

Since its identification as a homologue of TRX, MLL has been knocked-out in mice. Results confirmed that MLL is not only homologous to TRX in structure but also in function (Fig 2.1). Mice lacking a functional MLL die at E10.5 days, and show anterior skeletal transformations (Yu et al., 1995; Yu et al., 1998; see Section 1.3.5). Likewise, in *Drosophila*, null mutants of TRX are embryonic lethal and display an anterior transformation of body segments (Ingham, 1998; see Section 1.3.5).

Despite these early studies, the function of MLL still remains elusive. The only insight into how MLL may possibly function in mammals was deduced from *Mll* -/- mice and has been extrapolated from studies of TRX by taking advantage of their conservation. MLL and TRX have been shown to be important for maintaining an open chromatin configuration since animals lacking TRX or MLL function result in repression of homeotic gene boundaries, and consequently, an anterior body transformation is observed (Ingham, 1998; Yu et al., 1995; Yu et al., 1998). TRX is bound at its regulatory elements dynamically throughout early

Drosophila development (Orlando et al., 1997). It is postulated that many different TRX complexes exist that differentially regulate its target genes since several types of TRX mutants are able to survive past embryogenesis into larval to pupal stages (Breen, 1999). However, different combinations of *trx* alleles have rendered flies that are viable and that are often associated with anterior transformation of body segment (Breen, 1999). In addition, two conserved domains, the SET domain and PHD fingers, which are shared between TRX and MLL and several other PcG/trxG members are thought to play an important role in TRX function. Interestingly, it is worth to note that MLL fusion proteins found in leukaemic patients contain an altered PHD finger, often missing at least half of the domain, and completely lacking the SET domain.

Several attempts have been made to create a mammalian model system to study the function of MLL and its fusion proteins identified in leukaemic patients. To date, the only surviving mouse harbouring an MLL fusion construct is MLL-AF9 (t(9:11)) (Corral et al., 1996) These mice are viable and develop leukaemia within 4-12 months. Attempts to create MLL-AF4 (t(4:11)) mice have been unsuccessful (E. Canaani, personal communication). Due to difficulties in generating transgenic mice and in manipulating mice genetically, the field has relied heavily on cell culture systems to study the behaviour of cells lacking MLL wild-type function. However, tissue culture usage is limited since cells already harbour inherent mutations allowing continued proliferation. To study specifically how MLL fusion proteins contribute to leukemogenesis in tissue culture becomes extremely complicated when considering the unaccountable influence inherent mutations may have on cell behaviour.

In this respect, a more suitable model system that can be implemented to study MLL fusion protein function per se is *Drosophila melanogaster*. The advantages of using *Drosophila* as a model system rely on its well-documented genetics and on the availability of the full genomic sequence. Genetic screens can be performed to identify interacting proteins that can modulate a specific phenotype. Most importantly, proteins selected through a screen function in a complex chromatin and in a multi-cell environment, a scenario more relevant for cancer research. The identification of proteins is then easily accomplished by using BLAST analysis against the attained sequences. Finally, the expression of MLL fusion proteins in *Drosophila* allows the study of the function associated with only MLL fusion proteins in the

absence of any influence from additional mutations as found in cell cultures and in primary tumour cell lines isolated from patients.

The fact that conservation exists both in PcG/trxG families (Section 1.3.5) and in the immune systems (Section 3.1) between *Drosophila* and mammals provide an ideal situation to establish *Drosophila* as an additional model system to study MLL fusion proteins. Chapter 2 focuses on presenting data validating *Drosophila* as an additional tool to study MLL fusion function. The results indicate that MLL fusion proteins specifically interfere with proper *Drosophila* development causing lethality, while MLL expressing flies are completely viable. Lethality is manifested when the fusion proteins are expressed in the blood cells and in neural cells. Moreover, this lethal phenotype is not a result of the mere presence of the fusion proteins since crosses with several Gal4 driver lines generate healthy, viable flies. The results are discussed in detail in relation to known phenotypes of MLL fusion proteins expressed in the mouse and to symptoms of patients harbouring the specific MLL translocation.

2.2 Results

2.2.1 MLL and TRX share limited homology, whereas the human MLL C-terminal partners are conserved in *Drosophila*.

Over 30 partner genes have been identified to fuse with MLL to cause over 80% of infant leukaemia. Two of these fusion proteins were selected for their expression in *Drosophila*. The MLL-AF4 (t(t11:4)) translocation was chosen because over 40% of children with leukaemia harbour this translocation. The prognosis of MLL-AF4 derived lymphoblastic leukaemia is very poor, as survival rates are less than 25% by the age of 5 years. The second chimeric protein expressed in *Drosophila* is MLL-AF9 (t(11:9)). MLL-AF9 is also widely mutated in leukaemia and is generally associated with 27% of myeloid derived leukaemia in infants. Mice expressing MLL-AF9 have been generated and have been observed to develop leukaemia, recapitulating the human case (Corral et al., 1996). Figure 2.1 depicts a schematic representation of the constructs used to generate the transgenic fly lines.

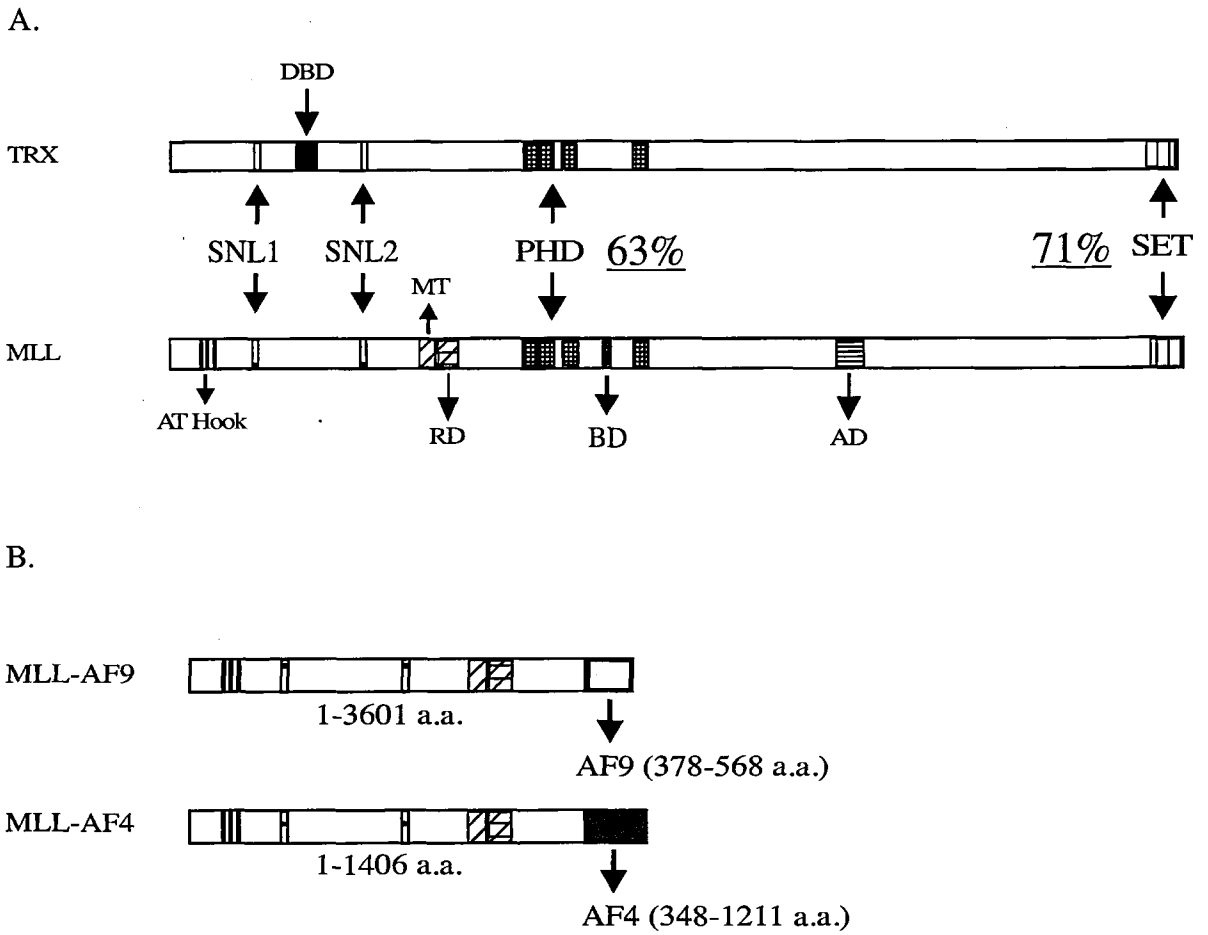


Figure 2.1. A schematic representation of TRX, MLL and MLL fusion proteins. (A) TRX and MLL share homologies mainly at the PHD fingers and the SET domain. BLAST analyses between the full cDNA sequences of TRX and MLL reveal that at the nucleotide level, the PHD fingers are homologous at 63% while the SET domain shares 71% homology. Additional homologous regions were not identified by BLAST analysis. (B) The two MLL fusion proteins expressed in the fly are MLL-AF9 and MLL-AF4. Both translocations and expression of fusion proteins have been identified most frequently in acute lymphoblastic or myeloid leukaemia. The AF9 and AF4 C-terminal portions of the fusion proteins are characterised by rich proline and serine stretches, which have been postulated to mediate protein-protein interaction (Nakamura et al., 1993). SNL: speckled nuclear localisation; DBD: DNA binding domain; MT: methyltransferase homology; RD: repression domain; PHD: plant homology domain; BD: bromodomain; AD: activation domain; SET: Su(var)3-9, E(z), TRX homology domain.

The functions of AF9 andAF4 in mammals are not known to date. Since at least one putative homologue for AF9 has been identified in *Drosophila* by the Berkeley Drosophila Genome Project, the implication here is that a homologous AF9 activity exists in *Drosophila* (Adams et al., 2000). The generation of an AF9 mutant in *Drosophila*, which is more feasible than in a mammalian system, would provide the first clues as to what this gene does. Furthermore, the reports of a *Drosophila* homolog for AF4, *lilliputian*, has implicated *lilliputian* in the regulation of cell growth in *Drosophila* (Adams et al., 2000; Tang et al., 2001; Wittwer et al., 2001). The presence of analogous proteins in *Drosophila* supports the continued dogma that partner genes fused with MLL are also conserved (Fig. 2.2; Rowley, 1998).

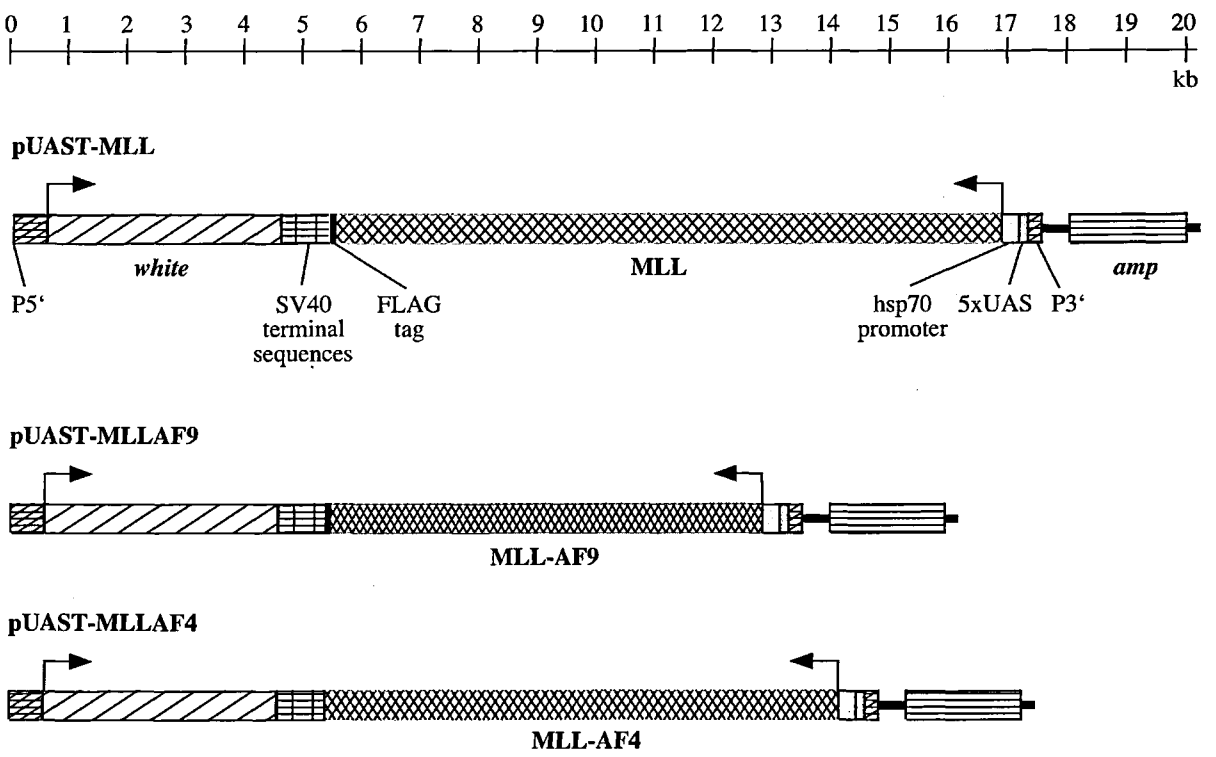
Protein Name	Score P(N)	Accession No.	Cytological Location
lilliputian *(homolog to human AF4)*	5.1×10^{-9}	Q9VQI9	23C1-2
ENL/AF9 *(homolog to human AF9)*	7×10^{-60}	Q9VF92	88E11-13
CG9207 *(homolog to human AF9)*	7.3×10^{-11}	Q9VM63	27C4-7

Figure 2.2. BLAST searches for homologous proteins in Flybase for AF4 and for AF9 reveal that *Drosophila* and mammals share similar regulatory pathways. Protein sequences for the human AF4 and AF9 sequences were submitted for BLASTP analysis against the predicted protein sequence of the *Drosophila melanogaster* genome. Both AF4 and AF9 have homologs in *Drosophila* as reported by the recent completed sequence of the fly genome (Adams et al., 2000). Interestingly, both AF4 and AF9 are conserved across species, where homologous proteins in *C. elegans*, *S. cerevisiae*, *S. pombe*, and *M. musculus* have been reported, indicating that the pathways through which these proteins function, are likely conserved. P(N): statistical probability that two given sequences are randomly similar; Accession No.: the associated number given to the identified gene in Flybase; Cytological location: the location of a given gene on polytenes.

2.2.2 Steps involved in the generation of MLL, MLL-AF4, and MLL-AF9 transgenic fly lines.

A specific P-element *Drosophila* vector, pUAST, was utilised to clone the MLL, MLL-AF9, and MLL-AF4 sequences as described above. The clones were generated as a collaborative effort by Tanya Rozovskaia from the laboratory of Eli Canaani at Weizmann Institute, Rehovot, Israel. At least three clones were created and sequenced at the N-terminus of the protein sequence to confirm that the open reading frame was intact. Restriction enzyme analysis reaffirmed that the correct sequences were properly cloned (Fig. 2.3).

A.



B.

Numbers	Lanes	Expected Band Sizes (bp)
1	Roche MW VI	21226, 5248, 4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, 564
2	Roche MW III	2176, 1766, 1230, 1033, 653, 517, 453, 394, 298, 234/220, 154
3	MLL *Stu I/ BamHI*	7749, 3218, 2837, 2137, 2076, 1293, 859, 700, 30, 12
4	MLL *Stu I*	9042, 6557, 3260, 2076
5	MLL-AF4 *Stu I/ BamHI*	7749, 2837, 2700, 1292, 1256, 12
6	MLL-AF4 *Stu I*	9041, 4093, 2712
7	MLL-AF9 *Stu I/ BamHI*	7749, 2837, 1460, 1325, 12
8	MLL-AF9 *StuI*	9042, 4337

C.

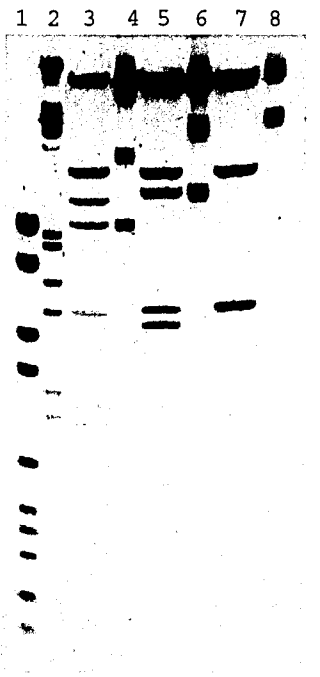


Figure 2.3. Positive clones of pUAST-MLL, pUAST-MLLAF4, and pUAST-MLLAF9 were verified by restriction digests. Diagnostic restriction enzyme digests with either Stu I or with Stu I and BamH I revealed that MLL, MLL-AF4, and MLL-AF9 were properly cloned into pUAST. (A) A schematic representation of the pUAST constructs used to make transgenics. Protein expression requires the binding of Gal4 onto the five UAS regulatory elements. The *white* gene is used as a selection marker to detect which flies have integrated the pUAST construct into the genome, whereas *ampicillin* resistance facilitates propagation of the pUAST plasmid. (B) Expected band sizes after the indicated digestion. (C) Electrophoresis gel showing that the pUAST constructs digested as expected.

pUAST-MLL, pUAST-MLL-AF9, and pUAST-MLL-AF4 were microinjected into w^{1118} embryos harbouring a mutation at the *white* locus. Around 30-50% of the embryos survived and were then crossed to Bcgl/Cyo virgins or males (Fig 2.4). Bcgl/Cyo marks the second chromosome of *Drosophila* and was used to map which chromosome the pUAST construct integrated. Progeny from this cross were screened for the presence of *white* expression, which was used as a transformation marker. At least 5 randomly integrated independent transgenic lines for each P-element pUAST construct were obtained and were maintained as separate stocks.

pUAST Construct	No. of w^{1118} embryos injected	Percentage of hatched embryos	No. of Transgenic Fly Lines generated
MLL	339	50.4%	7
MLL-AF4	528	36.9%	8
MLL-AF9	488	55%	7

Figure 2.4. Injection of w^{1118} embryos with pUAST-MLL, pUAST-MLLAF4, and pUAST-MLLAF9, recovered 7-8 independent lines. Two lines of each construct were selected for further experiments.

2.2.3 MLL fusion proteins induce larval to pupal lethality in *Drosophila.*

To identify phenotypes that are induced by the MLL fusion proteins (designated as MLL-AF4 or MLL-AF9) and/or by full-length MLL (referred to as MLL), the UAS-Gal4 system was employed. The UAS-Gal4 system adapts the yeast Gal4 as an intermediary protein for the expression of protein sequences juxtaposed to the UAS (Upstream Activating Sequence), which allows transcription upon specific binding by GAL4 (Brand and Perrimon, 1993; Fig. 2.5). Two separate fly lines are required for this system: one expressing the Gal4 protein, or the "Gal4 driver" , and a second containing the appropriate pUAST fly line. The advantage of using the system is that protein expression can be limited to specific tissues and to desired time windows.

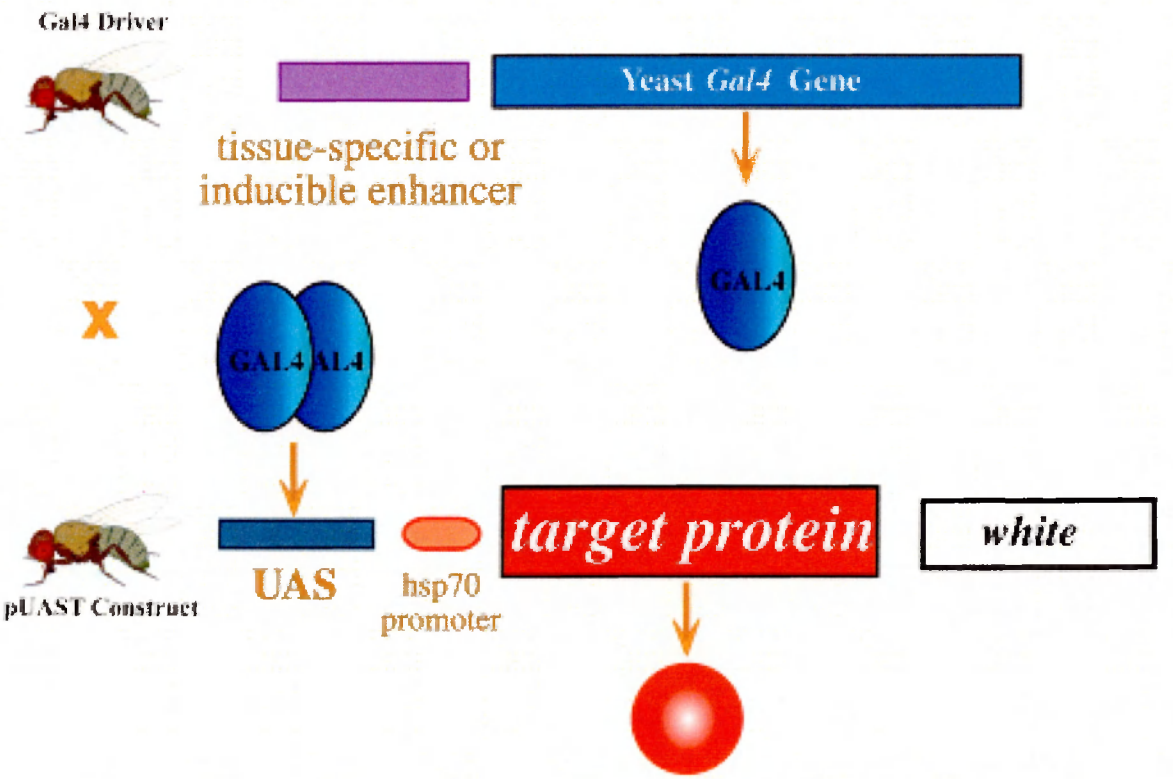


Figure 2.5. The UAS-Gal4 system. The controlled expression of a desired transgenic protein ("target protein") is achieved by crossing a fly line harbouring the pUAST construct encoding the "target protein" with a Gal4 driver line. The Gal4 driver consists of a tissue specific promoter or inducible enhancer element, upstream to the yeast Gal4 gene. The promoter or enhancer element limits Gal4 expression to specific tissues at defined times in development depending on the element's natural activity. The expression of the "target protein", which is under the control of the Gal4 UAS regulatory elements and a minimal heat shock promoter, requires the binding of Gal4 to UAS. Therefore, the "target protein" expression is defined by the expression profile of Gal4.

MLL, MLL-AF9, and MLL-AF4 transgenic lines were crossed to several Gal4 drivers, and progenies were then analyzed for phenotypes induced. All Gal4 drivers used to express MLL resulted in completely normal, viable flies. On the other hand, expression of MLL-AF4 and MLL-AF9 caused larval stage 2 to pupal lethality when expressed ubiquitously in the fly (Fig. 2.6). Interestingly, it was noted that the time of development was significantly prolonged for MLL-AF4 and MLL-AF9 transgenic lines. At 29 °C, *Drosophila* requires 8 days to develop from an embryo into an adult fly. Flies expressing MLL fusion proteins enter larval stages like wild-type and MLL expressing flies; however, at larval and pupal stages, development extends over time (Fig 2.6). This phenomenon has never been observed for flies expressing full-length MLL.

It is important to note that for all crosses, at least two independent lines were used. The reason for this precaution is to confirm that indeed the phenotype induced is not a consequence of the insertion site of the pUAST vector during microinjection. Additionally, because gene expression varies according to the chromatin environment at the site of integration, the severity of the lethal phenotype can be correlated with transgenic protein expression levels. It has been established that the extent of red eye pigment reflects the accessibility Gal4 has to the UAS regulatory element (Brand and Perrimon, 1993). Accordingly, for each transgenic construct, an earlier lethality is observed for fly lines with darker eye pigments (such as MLL-AF9-T2 and MLL-AF4-T1G in Fig 2.6). Western blot analysis, using specific antibodies, which recognise the N-terminus of MLL, confirmed that indeed differential levels of protein expression exist, and that these levels correlate with the severity of the lethal phenotype (Fig 2.7).

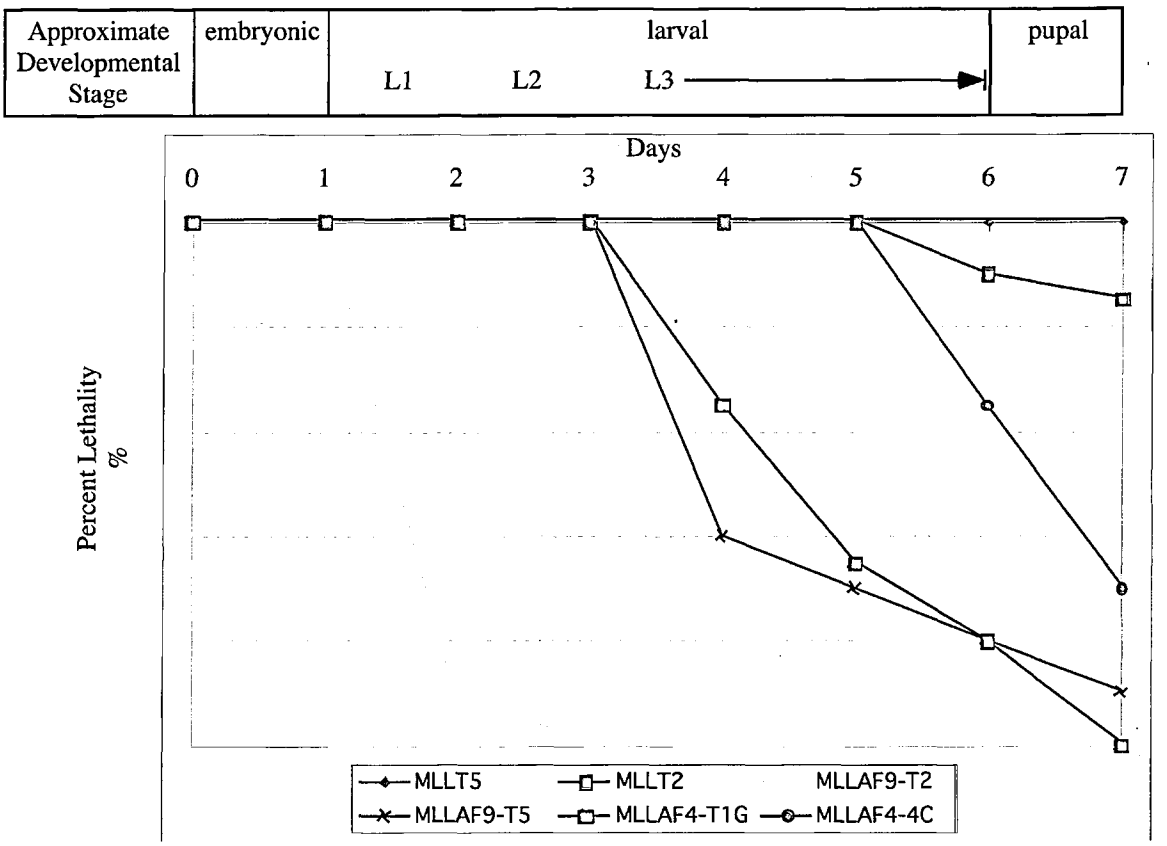
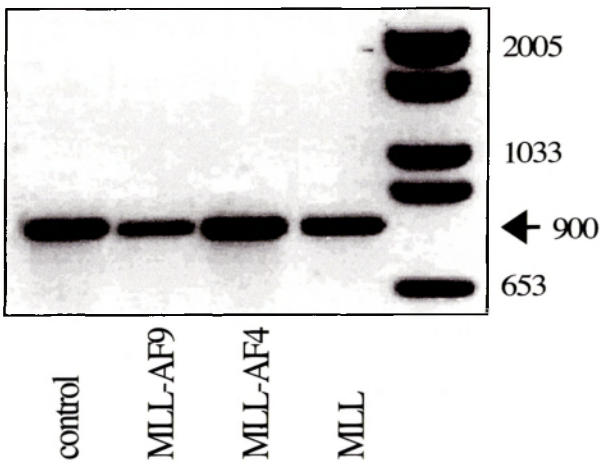


Figure 2.6. MLL fusion proteins, but not full-length MLL induce lethality in *Drosophila melanogaster*. To determine what kind of effect MLL, MLL-AF4, and MLL-AF9 may have on fly development, expression of the transgenic proteins by a strong Gal4 driver was used. The daughterless Gal4 (da Gal4) driver was selected since Gal4 expression is induced in high levels and begins at embryogenesis lasting throughout development. Transgenic flies, harbouring the pUAST-MLL, the pUAST-MLLAF4, or the pUAST-MLLAF9, were crossed to the daughterless Gal4 driver at 29 °C, and progenies were analysed for developmental malformation(s). Strikingly, MLL-AF4 and MLL-AF9 were found to strongly inhibit completion of *Drosophila* development, by apparently interfering with later developmental pathways. It was observed that embryogenesis proceeded like wild-type, whereas a delay in larval and early pupal development was observed. MLL-AF4 and MLL-AF9 expressing flies eventually died at larval to pupal stages. Importantly, this remarkable phenotype was never observed significantly with MLL expressing transgenic lines. Although MLLT2 displayed a lethality of 20% at late pupal stages, control crosses (w^{1118} x da Gal4), were also noted to exhibit a similar percentage of lethal pupae.

A.



B.

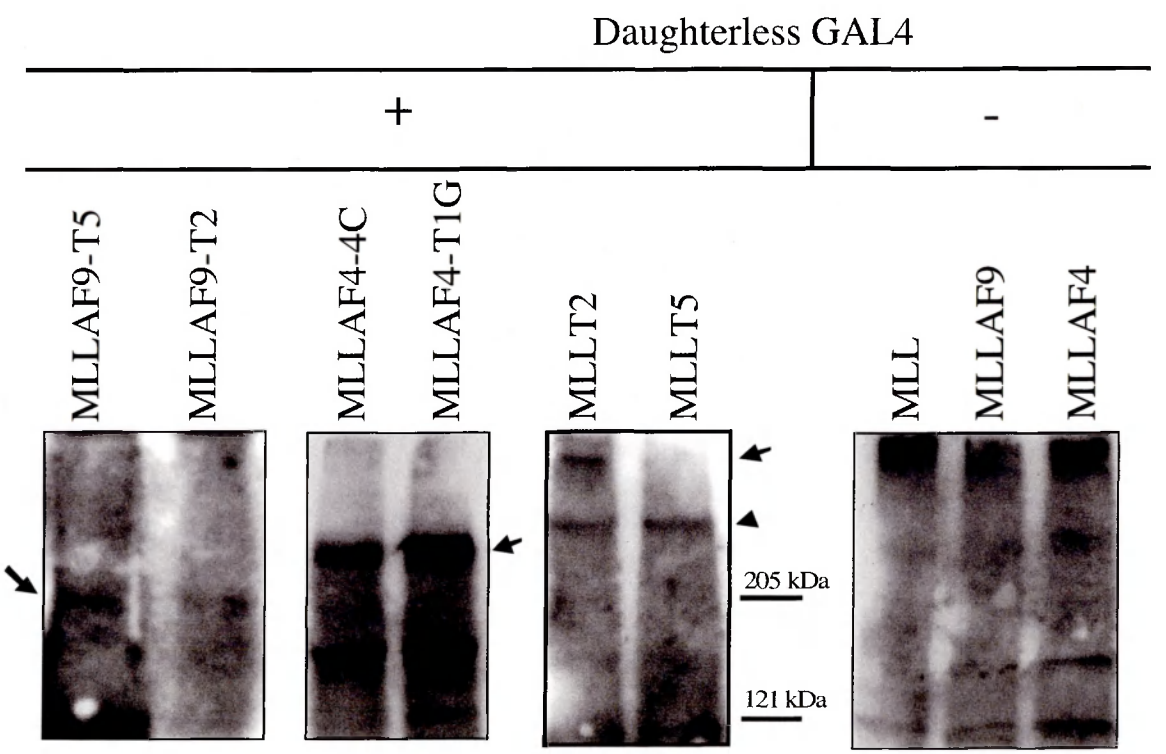


Figure 2.7. Transgenic proteins are expressed both at the RNA and at the protein levels. MLL, MLL-AF4, and MLL-AF9 were expressed with the daughterless Gal4 driver at 29 °C. (A) As shown, RT-PCR specific against the N-terminus of the MLL gene demonstrated that the complete ORF was being transcribed. (B) Western blot analysis revealed that the appropriate proteins, corresponding to the approximate expected transgenic protein sizes (MLL: 437 kDa; MLL-AF4: 250 kDa; MLL-AF9: 171 kDa), were detected from 20 dissected imaginal discs isolated from 3rd instar larvae in the presence of Gal4. The bands were found to be absent in fly lines harbouring the pUAST constructs alone (right). Arrows point to the specific transgenic protein band corresponding to the protein indicated above the lane, whereas the protein band defined by the arrow head has been consistently observed in MLL Western blots (R. Slany, unpublished results).

2.2.4 Directed expression of MLL fusion proteins at late developmental stages, in blood or in neuronal cells is sufficient for lethality.

To uncover additional effects caused by MLL and/or by MLL fusion proteins in *Drosophila*, a series of Gal4 driver lines were selected. These Gal4 driver lines possess special qualities that limit protein expression to specific tissues or to defined time in development. All crosses were performed in three different temperatures, at 18 °C, 25 °C, and 29 °C. The increase in temperature has been documented to cause higher protein expression levels due to a rise in Gal4 levels (Brand and Perrimon, 1993). Importantly, in all Gal4 drivers used to express MLL-AF9 and MLL-AF4, progenies were either normal or were lethal, indicating that MLL fusion proteins specifically and strongly alter an important pathway(s) necessary for completion of development. Figure 2.8 summarises the Gal4 driver lines used and indicates whether the lethal phenotype is observed.

Three different classes of Gal4 driver lines were tested for the lethal phenotype. The first category expresses the desired protein only at early embryogenesis (Fig. 2.8B). This is of particular interest since TRX and MLL are required to maintain appropriate gene expression during early development. In principle, MLL and/or MLL fusion proteins could prevent proper maintenance of gene expression patterns by TRX during embryogenesis. However, the lethal phenotype was never observed in any of the transgenic lines. This result indicates that MLL-AF9 and MLL-AF4 are not altering the classical pathway of TRX, involving the maintenance of early gene expression patterns.

The second class of Gal4 drivers used has been collectively termed late expressing lines (Figure 2.8B). These Gal4 drivers induce expression from late embryogenesis and throughout development. GawB is a constitutive promoter, which becomes active in all imaginal discs (Wilder and Perrimon, 1995). Act5C was isolated from the actin promoter and allows expression throughout development, peaking at late larval and pupal stages (Ito et al., 1997). Daughterless is a maternal protein ubiquitously expressed throughout development (Cronmiller and Cummings, 1993). Expression of MLL-AF9 and MLL-AF4, but not of MLL, under the control of these three drivers resulted in lethality. Manifestation of lethality correlated with the presence of proliferating tissues, which in *Drosophila* occur primarily in

imaginal discs at larval stage. These results, in conjunction with those involving early expressing lines, suggest that MLL-AF9 and MLL-AF4 interferes with a TRX-dependent or -independent function operating during late stages of development.

The third class of Gal4 drivers examined, were tissue specific. The MZ1580 driver, which expresses Gal4 in blood cells, induced lethality in MLL-AF9 and MLL-AF4 transgenic lines (Figure 2.8B). The MZ1580 driver was initially isolated in an enhancer screen for Gal4 expression in glial cells (Hidalgo et al., 1995). It was subsequently noted that this driver induces expression in macrophages (hemocytes). The latter comprises most of the *Drosophila* immune system. Gal4 expression commences at stage 11 when the first macrophages are released from the mesoderm of the procephalon. Following embryogenesis, Gal4 expression by MZ1580 becomes ubiquitous (Figure 2.9). When MLL-AF9 and MLL were driven by MZ1580, MLL-AF9 and MLL-AF4 alone, and not MLL, induced a severe lethal phenotype.

Using a Gal4 driver, gcm (glial cell missing) Gal4 which limits the expression of MLL and MLL fusion proteins at macrophages only (Bernardoni et al., 1997), revealed that MLL-AF9 and MLL-AF4 could not induce the full lethal phenotype, although partial lethality was observed. No effect of MLL expression by this gcm Gal4 driver on fly development was observable. The outcome of these experiments suggested that MLL fusion protein expression confined only to macrophages is not sufficient to completely inhibit fly development, again supporting the idea that expression of MLL fusion proteins in additional tissues at late development is an important event to recapitulate the late lethal phenotype. In this respect, it is interesting that the lethal phenotype was also observed when MLL-AF9 and MLL-AF4 were expressed in neural cells using the elav Gal4 driver, which sustains strong protein expression throughout development (Yao and White, 1994).

Taken together, the strong biological effect conferred by MLL-AF9 and MLL-AF4, together with the failure to find any effect associated with expression of normal MLL, supports the notion that *Drosophila* is a physiologically relevant system to study the role of MLL fusion proteins in a whole organism setting.

A.

Gal4 Drivers	Promoter	Expression Profile: Tissue & Time in Development
V2H/ V37	α-4 tubulin	early development expression
228 Gal4	unidentified enhancer	weak ubiquitous expression
Ap-Gal4 / Cyo	apterous	strong expression in the dorsal compartment of the wing
gl- Gal4	glass	strong expression in the eye after morphogenic forrow

B.

Expression Profile: Tissue & Time in Development	Gal4 Drivers	18 °C			25°C			29 °C		
		MLL	MLL-AF4	MLL-AF9	MLL	MLL-AF4	MLL-AF9	MLL	MLL-AF4	MLL-AF9
<u>early expressing lines</u>										
early expression	armadillo Gal4/CyO	✓	✓	✓	✓	✓	✓	✓	✓	✓
early ubiquitous expression	en2.4Gal4 / Sm5, Cy	✓	✓	✓	✓	✓	✓	✓	✓	✓
<u>late expressing lines</u>										
ubiquitous expression in all imaginal discs	GawB/ Cyo	✓	✓	✓	✓	†	†	✓	†	†
expression throughout development (late larvae & pupae peak)	Act5c-Gal4/Cyo	✓	✓	✓	✓	†	†	✓	†	†
"ubiquitous" expression	daughterless-Gal4	✓	✓	✓	✓	✓	+/-	✓	†	†
<u>tissue specific lines</u>										
hematopoetic system (stage 11)	MZ1580 (X)	✓	†	†	ND	ND	ND	✓	†	†
macrophage specific (stage 11)	gcm/ Cyo	✓	✓	✓	✓	✓	✓	✓	✓	+/-
neuron specific	elav Gal4	✓	✓	✓	✓	†	+/-	✓	†	†

Figure 2.8. Directed expression of MLL fusion proteins at late developmental stages and at neuronal and blood cells is sufficient to induce the lethal phenotype. MLL, MLL-AF4, and MLL-AF9 transgenic flies were crossed to several Gal4 drivers expressing Gal4 at different times and at specific tissues during development. (A) Several Gal4 driver lines used to express the transgenic proteins resulted in no observable phenotype, indicating that the presence of the exogeneous proteins is not toxic to the flies. (B) While early expression of MLL-AF9 and MLL-AF4 induced no lethal phenotype, expression of the MLL fusion proteins throughout or during late development caused fly lethality. Expression of MLL-AF9 and MLL-AF4 in the hematopoietic system or in neurons was also found to be sufficient for the manifestation of MLL fusion associated lethality. Importantly, lethality was never observed for MLL transgenic lines using any of the Gal4 drivers. (✓: viable, †: lethal, +/-: partial lethality (+/- 50%), ND: not determined)

Figure 2.9. The blood cell MZ1580 Gal4 driver expresses Gal4 ubiquitously after embryogenesis. Since the pattern of Gal4 expression by the MZ1580 enhancer has only been characterised in embryogenesis (Hidalgo et al., 1995), the pattern of Gal4 expression at later developmental stages was defined by crossing UAS-GFP with MZ1580. As shown, Gal4 expression by the undefined enhancer becomes ubiquitous throughout larval and pupal stages.

2.2.5 MLL cannot relieve late lethality induced by MLL-AF9 and MLL-AF4.

Since the expression of transgenic proteins by other strong Gal4 drivers did not affect larval or pupal development (Figure 2.8A), the lethal phenotype induced by MLL fusion proteins, when expressed at specific tissue and time in development (Figure 2.8B), is not due to a toxic effect by transgenic protein levels. To explore the relation between MLL and MLL fusion proteins on fly viability, stable lines containing several copies of one pUAST contruct or combinations of several pUAST constructs were generated. Since a higher amount of MLL fusion proteins correlates with a more severe lethal phenotype, as shown in Figure 2.6, increasing the copy number of MLL-AF4 or MLL-AF9 predicts an earlier stage lethality. As shown in Fig. 2.10, two copies of MLL-AF4 or MLL-AF9 induced lethality at pupal or larval stages, respectively. The similarity in phenotypes as shown in Figure 2.6 suggests that the lethal effect exhibited by the MLL fusion proteins can only be modulated to a certain extent, whereby additional amount of proteins has no effect.

To investigate if the presence of wild-type MLL can alleviate the lethal effect exhibited by MLL fusion protein expressing flies, by possibly interacting with MLL fusion proteins and thus creating non-functional entities, MLL was expressed with either MLL-AF9 or MLL-AF4. Effect on the lethal phenotype was not observable (Fig 2.10). The expression of MLL-AF9 and MLL-AF4 together also did not affect the severity of the fly lethality observed in flies expressing either MLL-AF9 or MLL-AF4 alone. This result implies that the MLL fusion proteins appear to not synergise in functional activity (Fig 2.10). Collectively, the results indicate that MLL fusion proteins interfere with fly development by acting in a dominant negative fashion, irrespective of full-length, and that MLL fusion proteins appear to target distinct pathways.

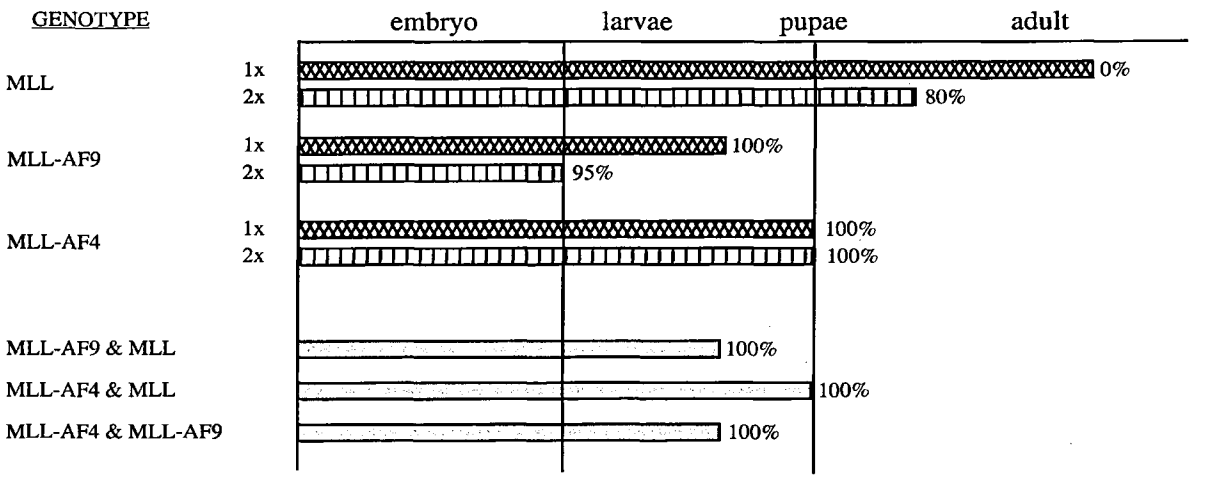


Figure 2.10. Additional expression of MLL or of MLL fusion protein does not alter the lethal phenotype. Stable stocks, containing several copies of MLL or of MLL fusion protein constructs, and of various combinations of MLL and MLL fusion constructs, were expressed with the blood MZ1580 and the late expressing Actin5c Gal4 drivers. Both Gal4 drivers were observed to induce the lethal phenotype with one copy of MLL-AF9 and of MLL-AF4 (Fig. 2.8), but not with one or two copies of MLL. Excessive copies of MLL-AF9 or MLL-AF4 did not alter the stage of the lethal phenotype, nor did flies expressing both MLL-AF4 and MLL-AF9 exhibit an earlier lethal phenotype. Presence of MLL with MLL-AF9 or MLL-AF4 could also not enhance fly viability. These results indicate that MLL fusion proteins act in a dominant negative fashion, irrespective of wild-type MLL, to inhibit vital pathways required for the completion of fly development, and appear to target separate pathways. Bars represent what stage progenies with the indicated genotype developed. Percentage numbers reflect the portion of progenies lethal at that stage in development (the remaining offspring was normal and viable).

2.3 Discussion

The results presented in Chapter 2 display a clear dichotomy in functional interference between MLL and MLL fusion proteins in *Drosophila* development. Apparently, MLL fusion proteins have gained additional functions possibly through their novel C-terminal sequences, which specifically inhibit or deregulate an essential pathway(s) necessary for *Drosophila* viability. Full-length MLL, on the other hand, has no effect in all conditions tested. Initially, the similar and severe phenotype induced by both MLL fusion proteins, MLL-AF9 and MLL-AF4, suggests that possibly, MLL fusion proteins deregulate a common, essential pathway necessary for cell survival at late developmental stages in *Drosophila*. This speculation is supported by the facts that (1) the presence of the transgenic proteins is not toxic, and (2) that all Gal4 drivers used to express MLL fusion proteins generated either normal or lethal progenies. However, the lack of a more severe lethal phenotype by the expression of both MLL-AF4 and MLL-AF9 argues for MLL-AF9 and MLL-AF4 targeting separate and important pathways necessary for the completion of development. The identification and subsequently dissection of the pathways with which these proteins interfere may potentially elucidate the contribution to leukemogenesis by MLL fusion proteins at the molecular level. In this context, *Drosophila melanogaster* would provide an invaluable tool for studying the functional roles MLL fusion proteins have acquired and/or have lost within a complex chromatin setting.

The finding that MLL fusion proteins can recapitulate the lethal effect by their presence in the blood system of the fly support the only published results in mice demonstrating that expression of MLL-AF9 induces cancer in myeloid, and to a certain extent, lymphoid blood cells, as in the human disease, and correlate with symptoms from human patients where aberrant MLL proteins are associated with leukaemia (Corral et al., 1996; Pui, 2000; Ayton and Cleary, 2001). The surprising result that expression of MLL fusion proteins at macrophages only cannot recapitulate the lethal phenotype suggests several points. First, MLL fusion proteins may not acquire the full capacity to deregulate the function of the immune system on their own, because of their inability to interact with conserved transcription factors regulating hematopoeitic differentiation (Chapter 3). Alternatively, low

transgenic protein levels induced by the weak expressing gcm Gal4 driver may be insufficient to induce complete lethality. In this respect, Figures 2.7 and 2.8 linked the severity of the lethal phenotype with transgenic protein levels. Second, and more likely, additional tissues expressing the fusion proteins are necessary for the manifestation of the lethal phenotype. In *Drosophila* immune system, the interplay between fat bodies and lymph glands/macrophages is necessary for appropriate immunological functions (Hoffman et al., 1999). The fat body has been documented to produce anti-microbial peptides, which aid in the recognition of "non-self" antigens (Meister et al., 1997; Ferrandon et al., 1998; see Section 3.1). The misregulation of peptide production causes a dysfunctional immune system (Meister et al., 1997). Therefore, expression of MLL fusion proteins only at macrophages by the gcm Gal4 driver may not be sufficient to disrupt regulation of the blood system. Indeed, GFP expression studies with the blood Gal4 driver MZ1580 shows that upon completion of embryogenesis, an ubiquitious expression pattern takes over throughout the remaining developmental stages. The fact, that lethality is observed in crosses with MZ1580, indicates that the expression of MLL fusion proteins in all imaginal tissues is an important event to acquire lethality. The finding that lethality is observed when Gal4 expression is sustained during later stages of *Drosophila* development underscores this point.

In addition to over-proliferation of blood cells, 3% of patients suffering from MLL-derived acute leukemia have been diagnosed to display extramedullary disease associated with the central nervous system (CNS) (Reaman et al., 1999; Dordelman et al., 1999). Failure to include specific therapy directed towards the CNS has resulted in the development of CNS leukemia in 50-70% of MLL patients (Pui et al., 1998). These reports indicate that MLL fusion proteins may have secondary tumourigenic effect beyond their effect on blood cell proliferation. In this respect, it is interesting that the *Drosophila* system has identified an additional pathway with which MLL-AF9 and MLL-AF4 interfere. The lethality observed in these studies, when MLL-AF9 and MLL-AF4 are expressed in neurons, may bear on the CNS disease.

Several models have been proposed to account for the existence of a heterogeneous MLL fusion protein population in leukaemia. At first, it was suggested that the N-terminal portion of MLL was crucial for leukemogenesis. However, the disruption of only the *Mll* gene

is not sufficient for the development of leukaemia as indicated by MLL-myc mice, which do not develop the disease (Corral et al., 1996). Most importantly, the study pointed to the C-terminal portion of the fusion protein as an entity conferring the chimera proteins tumourigenic. In support of this argument, the findings in the fly demonstrate that likely, the AF4 and AF9 C-terminal sequences enable the MLL fusion proteins to affect cell viability. Generation of Trx-AF4 and Trx-AF9 fusion proteins in *Drosophila* would validate if indeed only the C-terminal partner inhibits fly viability.

The study of MLL in *Drosophila* also comments on the relevancy of oligomerisation as a mechanism for oncogenesis. The recent publication of MLL-β-gal causing leukaemia in mice, and the demonstration that MLL's SET domain harbours the capacity to self-oligomerise and to oligomerise with ASH1 resulted in the speculation that oligomerisation between MLL fusion proteins with other components confers tumourigenic potential to at least some of the fusion proteins (Dobson et al., 2000; Rosenblatt-Rosen et al., 1998). The fact that MLL does not interfere with TRX function, suggests that oligomerisation between the SET domains appears to not have any dire effects on fly viability. In this respect, the results with the expression of MLL-AF9 and of MLL-AF4 in flies would argue for the specific interference(s) with vital pathways as a venue to achieve cell deregulation. Future experiments designed to express various constructs consisting of the N-terminal MLL fusion sequence, of AF9 and of AF4 C-terminal sequences, and of lack of the SET domain in both MLL and TRX sequences should elucidate more precisely the role of oligomerisation as functions of MLL leukaemic fusion proteins.

Intriguingly, the presence of MLL fusion proteins during early development was found to cause no developmental aberrations. If it is assumed that MLL N-terminal function remains intact and targets the chimeric proteins to MLL target genes during early development, it should be expected that proper maintenance of genes regulated by TRX is perturbed, assuming that TRX and MLL are interchangeable due to their conservation. The change in gene expression profile would be a result of dominant gain-of-function activities presumably originating from the C-terminus of the fusion protein. It is known that a critical step in development occurs where the switch between maternal and segmentation transcription factor regulation to PcG/trxG regulation occurs during embryogenesis. The lack of interference

observed with MLL fusion proteins at this level of regulation suggests that MLL fusion proteins appear to interfere with a TRX independent or a non-classical TRX pathway.

To date not much is known about how PcG and trxG members behave in later stages in development. It is forseeable that additional functions are mediated by PcG/trxG members during late development. A precedent in this respect is the well-established connection between Bmi-1, a PcG mouse homologue of Posterior sex combs (Psc), and ink4a-Arf, a cell cycle control gene (see Section 1.4.3). Over-expression of Bmi-1, as found in many cancers, causes the suppression of ink4a-Arf locus, which encodes two tumour suppressor genes. The potential that the *Drosophila* system can decipher a property similar to Bmi-1 for MLL in the regulation of cell cycle control is highly plausible and exciting. In this case, possibly, MLL fusion proteins alter a late TRX (and analogously, MLL in mammals) function required for the completion of *Drosophila* development (Chapter 4).

CHAPTER 3

MLL fusion proteins do not generate leukaemia or interfere with the apoptosis pathways in *Drosophila melanogaster*.

3.1 Introduction

The balance between cell proliferation and cell death has been postulated to be crucial during cell transformation (De Lorenzo et al., 1999). The remarkable discovery that insects are capable of developing neoplastic tissue demonstrated that the pathways controlling the regulation of cell proliferation are conserved across species. In addition, the ease with which insect models, especially the well-characterised *Drosophila melanogaster*, can be created to harbour mutations, allowed studies to elucidate the components required for neoplastic development. The pioneering studies, led particularly by the laboratory of Elisabeth Gateff, paved the way for the development of *Drosophila* as a model system to aid in the understanding of the mechanisms contributing to the onset of cancer (Gateff and Schneiderman, 1967).

The *Drosophila* model for cancer studies has identified several tumour suppressor genes, which were mutated in the human disease (De Lorenzo et al., 1999). The use of *Drosophila* genetics has also uncovered how different components relate to one another to abrogate cell integrity. The best example to date is the elucidation of *Dlg* (*discs large*), *lgl* (*lethal giant larvae*), and *scribble*, whose actions act on a common pathway to maintain apical-basal cell polarity and proper cell proliferation (reviewed in Wodarz, 2000). Specifically, all three proteins act on maintaining cell adhesion and are dependent on one another for correct localisation to septate junctions (Bilder et al., 2000). For epithelial-derived cancers, these results have large implications, suggesting that cytoskeletal organisation may aberrantly affect signalling components confined at cell junctions (Binder et al., 2000).

Mutations affecting blood proliferation and/or differentiation have also been studied extensively. The functional characterisations of these mutants are potentially insightful given the highly conserved hematopoetic systems between mammals and *Drosophila* . In mammals, the hematopoeitic system develops initially from the embryonic yolk sac and eventually diversifies into the myeloid and lymphoid cell lineages from stem cells in the bone marrow. Similarly, *Drosophila*'s hematopoietic system commences at embryonic stage 11, and is released from the procephalon to give rise to hemocytes, or blood cells. Both the mammalian

derived myeloid and lymphoid lineages as well as the *Drosophila* hemocytes are responsible for detection and disposal of invasive material, and use homologous transcription factors for their differentiation (Rizki and Rizki, 1981; Daga et al., 1996).

Simplistically, the immune response in mammals includes the recognition of non-self determinants by macrophages/monocytes, and the subsequent display of antigens to T-cells. T-cells in turn triggers specific immune response by activating B-cells for antibody production. The release of cytokines, triggered by activated macrophages, also help recruit and activate immune cells. In *Drosophila*, a similar response occurs. Identified non-self material is recognised by phagocytosing plasmatocytes or macrophages, and induce production of antimicrobial peptides, which facilitate recognition of invasive material. Encapsulation of the material occurs by the actions of lamellocytes, which aggregate around the pathogen. Somehow, activation of the crystal cells releases products of the phenoloxidase casacade which result in melanisation of the material (Ip and Levine, 1994). The ability of the *Drosophila* immune system to "remember" a foreign pathogen, as in the case in mammals, was remarkably demonstrated by Boman et al. (1972) who showed that flies injected with attenuated bacteria were protected against further infections.

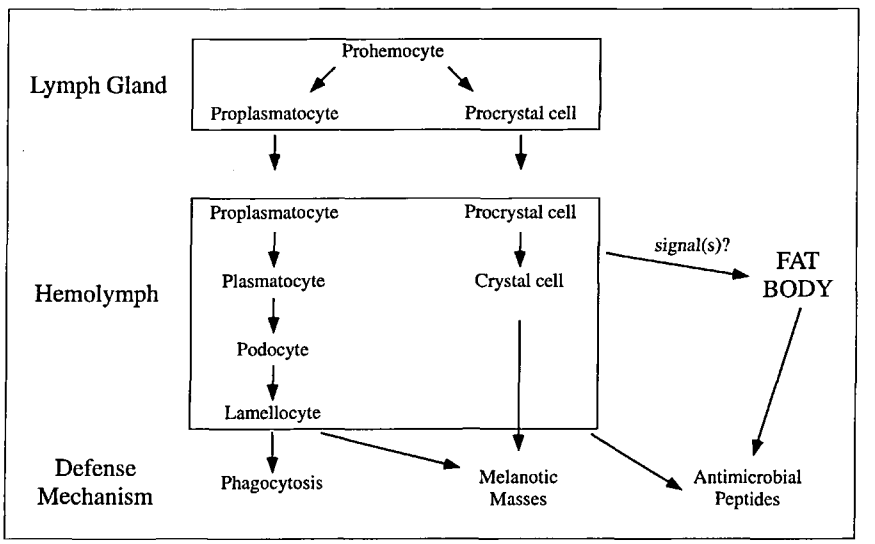


Figure 3.1. A schematic representation of blood cell differentiation in *Drosophila*. The precursors of *Drosophila* blood cells, or hemocytes, are developed in the lymph gland of the larvae. Precursors are released to the blood stream (hemolymph), which circulates the larval and adult body cavities. During an infection, the precursors differentiate into mature cells associated with specific immune functions. Plasmatocytes and podocytes are phagocytosing cells that first detect pathogens wheras lamellocytes encapsulate foreign material by aggregating around it. The crystal cells are responsible for releasing products of the phenoloxidase cascade that allow melanisation to occur. Antimicrobial peptides, produced in the fat body, are thought to enhance immune activity, by recognising "non-self" material. (adapted from Gateff, 1978; Mathey-Prevot and Perrimon, 1998; Hoffmann et al., 1999)

Three types of mutations affecting blood cell development have been reported. The categories include (1) mutations preventing blood cell proliferation (Gateff, 1994; Braun et al., 1998), (2) mutations allowing overgrowth of plasmatocytes and their differentiation into lamellocytes (Watson et al., 1991; Török et al., 1993), and (3) mutations inducing malignant neoplastic transformation of plasmatocytes (Gateff, 1978; Hou and Perrimon, 1997). The most striking mutations affecting the blood system include *domino*, which are devoid of hemocytes and which is similar to the "bloodless" phenotype of the mammalian null mutant for CBFA2/CBFB, and *hopscotch*, the only dominant *Drosophila* oncogene, which encodes the Jak kinase and which allows over 10 fold increase in plasmatocytes and premature blood cell differentiation at 29 °C (Braus et al., 1998; Binari and Perrimon, 1994; Hanratty and Dearold, 1993; Castilla et al., 1996; Okuda et al., 1996; Wang et al., 1996).

Interestingly, a member of the PcG family, *multi sex combs* (*mxc*), failed to complement the *lethal(1)malignant blood neoplasm* (*l(1)mbn*) mutation (Santamaria and Randhold, 1994). *l(1)mbn* is often considered as the prototype example of flies harbouring malignant blood cell tumours. *l(1)mbn* flies exhibit enlarged hematopoeitic organs, display constitutive expression of the antibacterial peptide diptericin, and are associated with 3-7 fold increase in blood cell counts and with a shift in blood cell differentiation from the hemolymph to the lymph glands (Shresta and Gateff, 1982a; Shresta and Gateff, 1982b). It is speculated that the inability of the numerous free plasmatocytes to recognise "self" from "non-self" cause invasion of larval tissues for phagocytosis by abundant lamellocytes and podocytes (reviewed in Gateff 1994). The creation of these aggregates, or melanomes, is thought to result in pupal lethality. The fact that *1(l)mbn* is *mxc*, has implicated PcG members in regulation of specific cell-types. Interestingly, in humans, several PcG and trxG members induce several types of hematopoeitic cancers when mutated (see Sections 1.4 and 1.5).

In addition to alteration in blood cell proliferation, mutations giving rise to tumours in the fly have been shown to be important for the integrity of chromatin structure. Two papers have demonstrated that some *Drosophila* tumour inducing lines are associated with defect in somatic pairing, as determined by polytene analysis (Riede, 1996). The remarkable formation of polytenes in the brain, which never occurs in the wild-type situation, has also implicated

these mutants to somehow induce DNA replication at S-phase of the cell cycle without mitosis. The net result is the detection of "polytene-like" structures in the brain (Reide, 1996).

Chapter 2 presented evidence demonstrating that MLL fusion proteins act in a dominant negative manner to cause larval to pupal lethality, and importantly, their expression at the hematopoeitic system of the fly is sufficient to induce lethality. The finding that *mxc* is implicated in deregulating blood cell homeostasis encouraged experiments directed at elucidating the effect MLL fusion proteins have on blood cell development. For this, blood cells from the hemolymph were isolated from larvae and were found to be relatively normal in number and in morphology, indicating that expression of MLL fusion proteins does not cause leukaemia in a wild-type background. Although it appears that blood cell proliferation is not affected by MLL fusion proteins, analyses of polytenes revealed that only MLL-AF4 and MLL-AF9 are associated with defect in polytene pairing, although polytene-like structures in the brain were found in MLL and in MLL-AF4, and surprisingly, not in MLL-AF9.

Since MLL fusion proteins have been implicated to inhibit or to induce apoptosis in tissue culture experiments (see Section 1.5.4), it is conceivable that fly lethality by MLL fusion proteins may reflect the inability of cells to die or to survive at correct times in development, and therefore, halt developmental progression. To address if MLL fusion proteins affect the apoptosis pathway, cell clones expressing the appropriate proteins were generated. Isolated imaginal discs, containing the clones, were subjected to the apoptosis TUNEL assay. Results support that MLL fusion proteins appear to not affect cell death program during development, and demonstrate that MLL fusion proteins behave differently in a complex, multi-cellular environment.

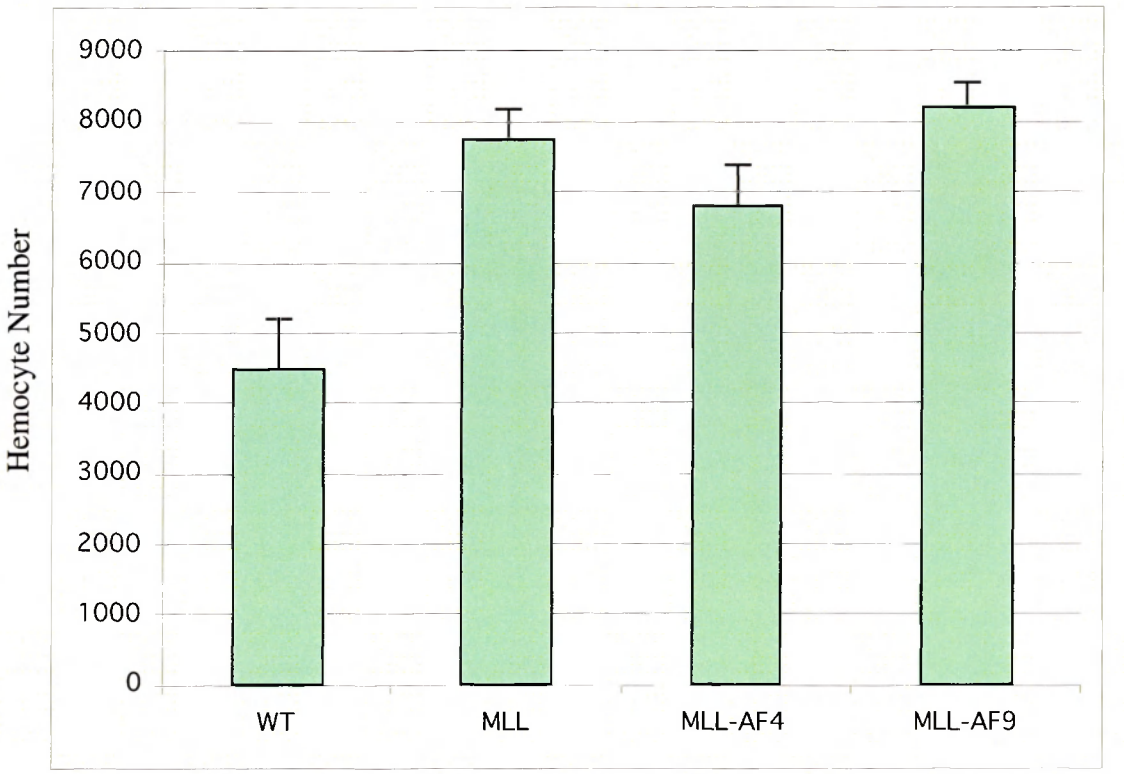
3.2 Results

3.2.1 Blood cells are normal in MLL and in MLL fusion chimera transgenic fly lines.

The hemolymph in *Drosophila* consists of hemocytes, which collectively comprise the blood system of the fly (Mathey-Prevot and Perrimon, 1998). Within the hemolymph, several types of blood cells are detected. In wild-type flies, the hemolymph consists largely of plasmatocytes. However, blood tumour mutants possess various blood cell types in the hemolymph under non-infectious conditions (Gateff, 1994). Blood cell types include lamellocytes and podocytes, which are responsible for encapsulating and phagocytosing invasive, "non-self" material. Normally, the differentiation of plasmatocytes into lamellocytes and podocytes occurs during larval infection. The finding of several classes of blood cells in the hemolymph of tumour mutants indicates that pre-mature differentiation has occurred.

To assess if MLL fusion proteins perturb the development of fly hematopoesis, hemolymph from 10 larvae from each transgenic lines was isolated. The hemolymph solution was placed on a drop of mineral oil on a hemocytometer, and hemocytes were counted. As shown in Fig 3.2, the numbers of hemocytes for each transgenic line were elevated as compared to wild-type. In addition, characterisation of the morphology of the hemolymph revealed that like wild-type only plasmatocytes were observed (Fig. 3.3). Differentiated cells, such as lamellocytes and podocytes, were not present. Taken together, these results demonstrate that the effect by MLL and by MLL fusion proteins alone on blood cell proliferation and blood cell differentiation, do not inhibit completion of *Drosophila* development.

A.



B.

Transgenic Fly Lines	Relative Hemocyte Enrichment (to WT level)
MLL	1.7
MLL-AF4	1.5
MLL-AF9	1.8

Figure 3.2. Hemocyte counts reveal that MLL and MLL fusion proteins equally affect blood cell proliferation. MLL, MLL-AF4, and MLL-AF9 were expressed using the MZ1580 blood cell Gal4 driver. (A) Hemolymph from 10 larvae for each transgenic fly line and for wild-type was isolated and counted on a hemocytometer. Average of blood cell counts reveal an equal effect on blood cell proliferation by MLL and by MLL fusion proteins. (B) Relative ratios of hemocyte counts between MLL transgenic lines and wild-type. These results indicate that the elevation of blood cell numbers do not correlate with MLL fusion protein induced lethality.

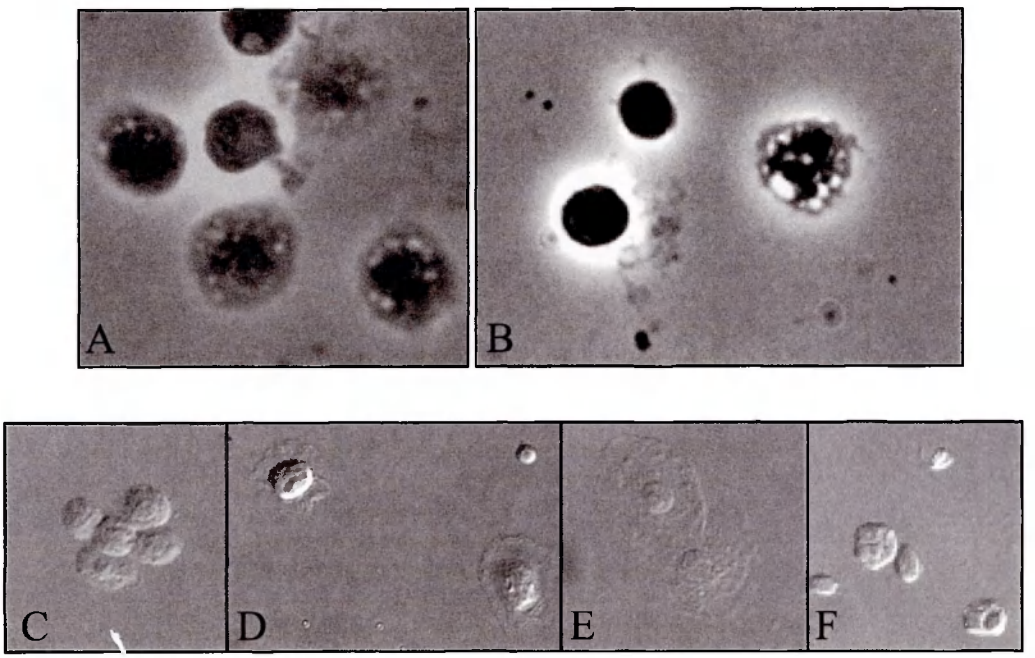


Figure 3.3. Blood cell differentiation is apparently unaffected in transgenic fly lines. MLL, MLL-AF9, and MLL-AF4 were expressed using the blood Gal4 line MZ1580. Under this condition, MLL fusion proteins induce larval and pupal lethality. Isolation of hemolymph from these larvae for all transgenic fly lines demonstrated that MLL, MLL-AF9, and MLL-AF4 expressing hemolymph comprised of plasmatocytes (B) as in the case of the viable Gal4 driver line, MZ1580 (A). Several blood types that have been documented in blood tumour fly lines are shown as follows: (C) plasmatocytes, (D) activated macrophages, (E) lamellocytes, (F) crystal cells (M. Meister, Strasbourg, France). None of these differentiated blood cells were observed in any of the hemolymph isolated from the transgenic lines.

3.2.2 Polytenes in the brain and somatic pairing defect in polytenes indicate that MLL fusion proteins affect chromatin integrity.

The reports that *Drosophila* brain tumour mutants displayed pairing defects of polytenes and possessed polytene-like structures in the brain suggested that for some tumour suppressor mutants, the compromise of chromatin architecture has influenced the cell cycle. Presumably, the presence of polytenes suggest that diploid cells in the brain must have replicated their genomes numerous times to allow detection of polytene-like strands at interphase of the cell cycle. Since MLL fusion proteins are associated with inhibiting the

function of a chromatin regulator (MLL/TRX), analyses of salivary gland polytenes and of brain squashes were performed with the aim to ascertain if the defects were present.

As shown in Figure 3.4, pairing defects were clearly present for MLL-AF9 and MLL-AF4 transgenic constructs, although MLL expressing fly lines were never observed to display aberrant polytene morphology. In addition, polytene-like structures were found in MLL-AF4 expressing brains, although not in MLL-AF9 (Fig. 3.5). Unexpectedly, detection of similar structures was also documented in MLL expressing fly lines. These results indicate that MLL fusion proteins may have a direct role in modulating chromatin architecture. However, effect on cell proliferation may not be a property conferred to all MLL fusion proteins.

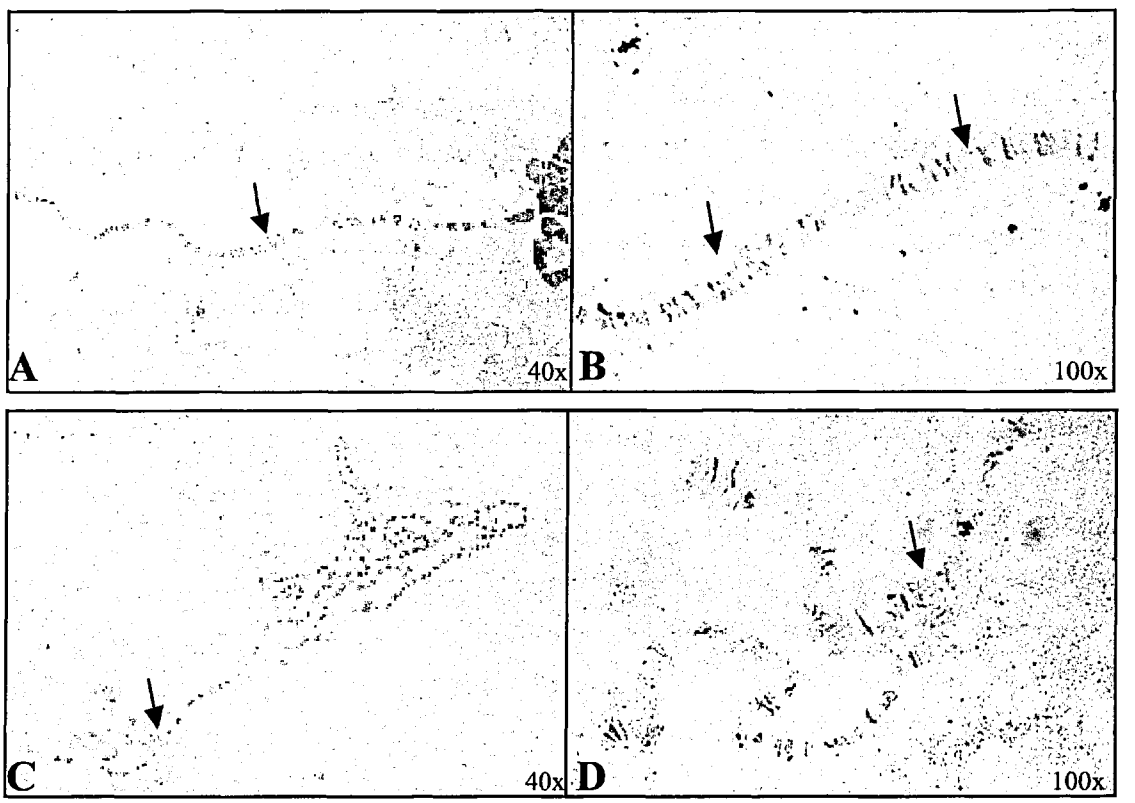


Figure 3.4. Pairing defect in polytenes is observed for MLL-AF4 and for MLL-AF9, but not for MLL. MLL, MLL-AF4, and MLL-AF9 were expressed using the daughterless Gal4 driver line, which allows the induction of the lethal phenotype associated with the MLL fusion proteins at 29 °C. Salivary glands were isolated from appropriate fly lines, were spread on slides, and stained with orcein. As shown, pairing defect was observed for MLL-AF9 (A: 40x, B: 100x) and for MLL-AF4 (C: 40x, D: 100x). These defects consist of splits along the arms of the chromosomes, as indicated by the arrows. However, splitting of chromosome arms, such as these polytene pairing defects shown, were not detected in full-length MLL and in wild-type.

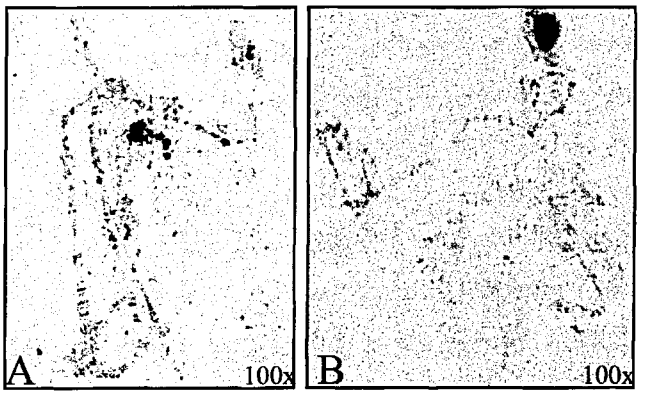


Figure 3.5. Polytene-like structures were observed in MLL and MLL-AF4, but not in MLL-AF9 expressing transgenic fly lines. MLL, MLL-AF4, and MLL-AF9 were expressed with daughterless Gal4 at 29 °C. Orcein-stained brain squashes with isolated brains from all transgenic fly lines revealed that polytene-like structures were present in MLL-AF4 (A) and in MLL (B) (1-2 spreads were observed per brain). Unexpectedly, such polytene-like structures were not observed in MLL-AF9.

3.2.3 TUNEL assay reveals that apoptotic pathways appear to be intact in MLL and in MLL fusion chimera fly lines during *Drosophila* development.

Tissue culture studies have implicated MLL fusion proteins in the interference of apoptosis (see Section 1.5.4). To exclude that fly lethality induced by the MLL fusion proteins is a consequence of misregulated apoptotic pathways, clones were generated expressing the transgenic proteins, and then subjected to the TUNEL assay.

Figure 3.6 depicts the strategy employed to generate transgenic protein expressing clones. The idea is based on the use of the FLP-FRT system. The FLP protein is a site-specific recombinase which functions at specific sites called FRT (Dang and Perrimon, 1992). The expression of FLP allows recombination between two FRT sites with the outcome that sequences between the two FRT sites are excised. Fly lines were created harbouring the pUAST expressing transgenic protein construct and the FLP protein under a heat shock inducible promoter. These stable lines were then crossed to a Gal4 driver composed of the

Act-5c promoter, the yellow marker flanked by FRT sequences, and the Gal4 open reading frame (AyGal4). A GFP marker under the control of the UAS enhancer has been recombined with AyGal4 to allow visualisation of the cells which expresses the Gal4 protein. This group of GFP positive cells (clones) also express the transgenic fusion proteins.

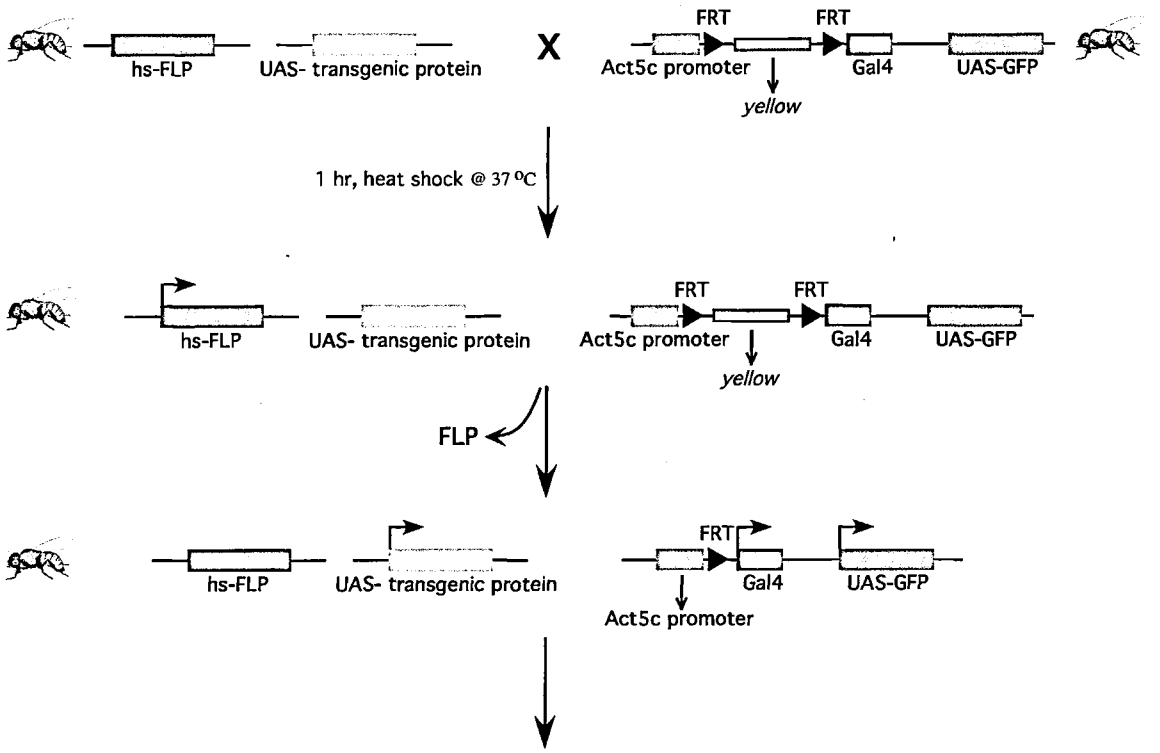


Figure 3.6. The FLP-FRT system. Stable stocks were generated containing two DNA elements (left): one that allows expression of the FLP recombinase upon heat shock (green), and the second, that expresses a transgenic protein under the UAS-Gal4 system (yellow) (also see Fig. 2.5). These generated stable stocks were then crossed to an inactive Gal4 driver line under the Actin5c promoter (right). The expression of the FLP recombinase is induced by heat shocking first instar larvae for 1 hour at 37 °C. The presence of the FLP recombinase induces excision of the *yellow* marker flanked by the FRT sequences. This excision allows the expression of Gal4 regulated by the Actin5c promoter, thereby generating clones of cells, which express GFP and the desired transgenic protein (both are under the control of UAS).

First instar larvae were heat shocked for 1 hour at 37 °C water bath to allow expression of the FLP recombinase. Heat shocked vials were then kept at 25 °C until third instar larvae developed. From these larvae, all imaginal discs were isolated. The imaginal discs possessed GFP positive clones indicating that the FLP-FRT system was functional. If indeed MLL

fusion proteins affect apoptosis, it is conceivable to expect changes in tissue morphology. If MLL fusion proteins inhibit programmed cell death, an outgrowth or an expansion of GFP positive tissues should be visible, causing abnormal imaginal disc morphology or large GFP positive clones relative to GFP negative areas. Alternatively, the induction of apoptosis by MLL fusion protein should cause underdeveloped imaginal discs containing little or no GFP positive cells. As shown in Figure 3.8, tissue morphology and clone size appeared normal.

To address the putative apoptotic role of MLL fusion proteins, TUNEL assays were performed in isolated imaginal tissues, and monitored for presence of GFP positive clones. The TUNEL assay is based on the detection of fragmented DNA, a hallmark for apoptotic cells (Robertson et al., 2000), by labelling DNA ends with fluorescein conjugated dUTP (Figure 3.7). Figure 3.8 demonstrates that for all transgenic lines, the number of apoptotic positive cells, as indicated by punctate red nuclear staining, were comparable in both GFP positive and negative cells. Therefore, results from the TUNEL assay demonstrated that the number of apoptotic cells were not enhanced or inhibited by MLL fusion proteins during *Drosophila* development.

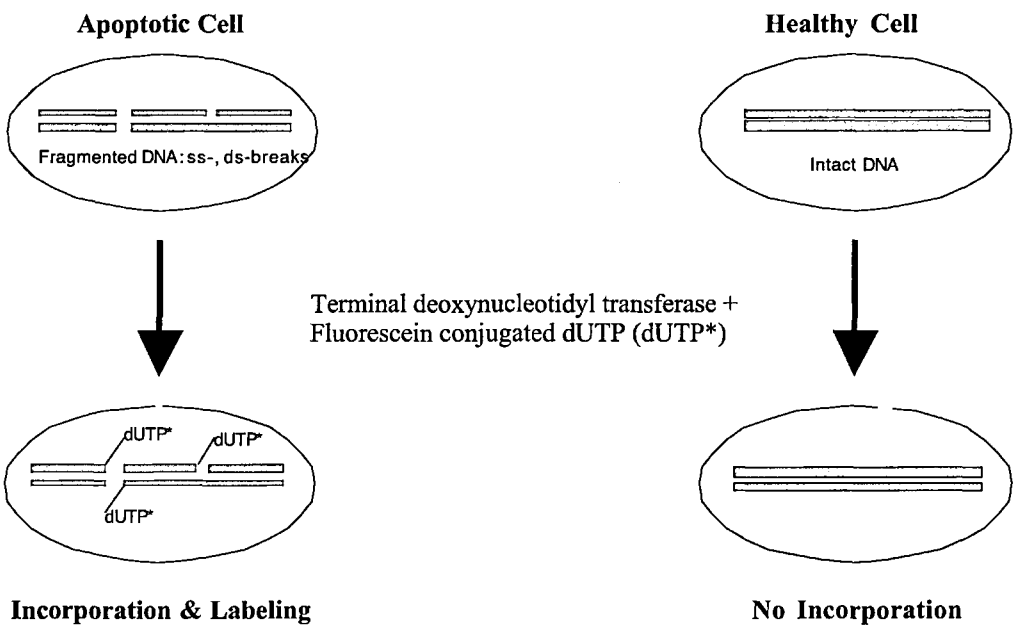


Figure 3.7. Schematic represenation of the TUNEL assay. Apoptotic cells are characterised by their fragmented genome. The TUNEL assay uses deoxynucleotidyl transferase to label ends of DNA with fluorescine conjugated dUTP. Signals from fluorescein conjugated dUTP are enhanced by using a secondary antibody against fluorescein. Positively labelled nuclei reflect cells undergoing apoptosis.

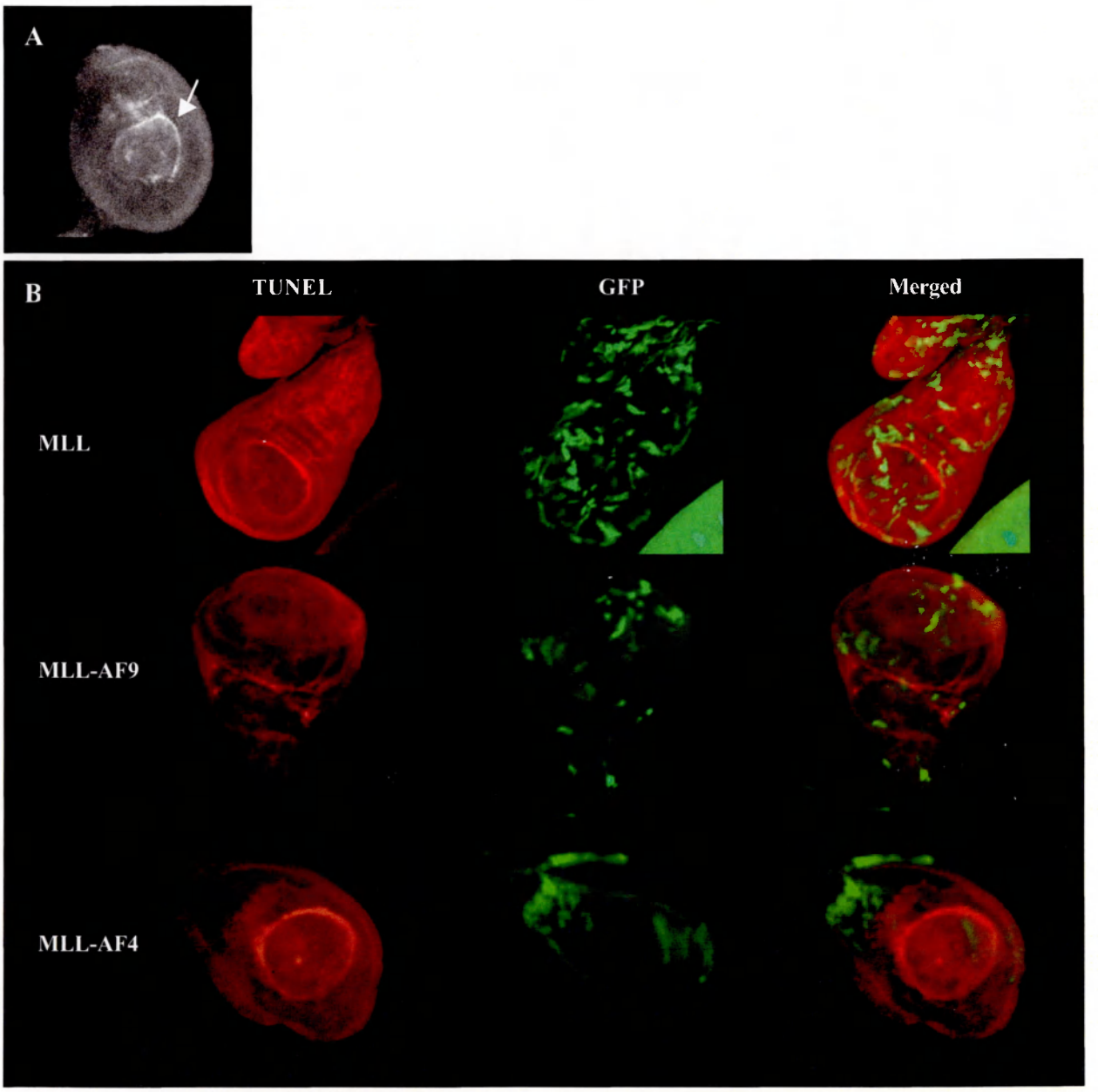


Figure 3.8. MLL, MLL-AF9, and MLL-AF4 do not affect apoptotic pathways during *Drosophila* development. (A) WT imaginal discs were subjected to the TUNEL assay. As shown for this leg disc, cells which are undergoing apoptosis during development exhibit positive nuclear staining (arrow). (B) MLL, MLL-AF9, and MLL-AF4 clones were generated using the FLP-FRT system. The clones, expressing the appropriate transgenic protein, are marked with GFP. Cells, which do not express the transgenic proteins, are GFP negative. Imaginal discs were isolated harbouring GFP positive clones and subjected to the TUNEL assay. The nuclear stainings (red) reflect cells that undergo developmental apoptosis. As shown, apoptosis (red nuclear staining) is not correlated with GFP positive clones.

3.3 Discussion

Experiments in Chapter 3 were aimed to explore possible pathways through which the MLL fusion proteins may cause larval to pupal lethality in flies. The association of MLL fusion proteins in deregulating hematopoeitic homeostasis (see Section 1.5) and in interfering with apoptosis in cell cultures (see Section 1.5.4), validated experiments directed at asking if such pathways have been compromised.

Studies in mammalian model systems have reported that MLL-AF9 affect myeloid differentiation (Slany et al., 1998; Dobson et al, 1999). The finding that expression of MLL-AF9, and of MLL-AF4, can ellicit the lethal phenotype when expressed at the hematopoeitic system of the fly suggested that MLL-AF9 and MLL-AF4 may affect blood cell differentiation and/or proliferation (Chapter 2). The result that isolated blood cells are similar in viable MLL, in lethal MLL-AF9 and in lethal MLL-AF4 transgenic flies, indicates that MLL fusion proteins' effect on blood cell differentiation or proliferation in *Drosophila* does not contribute to the induced lethality.

Although presumably MLL fusion proteins are expressed in all cells by the blood Gal4 driver MZ1580 late in development, the effect by MLL fusion proteins on blood cell development, may be masked by a dominant wild-type background. In this respect, *Drosophila* mutants, which are lethal at pre-pupae stage and are deficient for particular immune functions, still exhibit intact immune response. The isolation of *domino* mutants, which are devoid of blood cells, illustrates this point (Braun et al., 1998). Remarkably, the *domino* mutants are able to fight septic injury indicating that hemocytes (blood cells) are not required for this process. Only in a sensitised background (for instance, additional mutations affecting anti-peptide synthesis or melanisation) is defective immune response detected (Braun et al., 1998). Therefore, MLL fusion proteins may indeed have a greater dire effect on blood cell homeostasis, possibly by inhibiting hemocyte function, but which may only be detectable in a sensitised background. Crossing in several *Drosophila* immune deficient mutants to MLL fusion proteins may reveal their effect on blood cell homeostasis. In support of this idea, the *domino* gene has been recently reported to encode novel members of the SWI2/SNF2, which are DNA-dependent ATPases working on chromatin to regulate homeotic genes (Ruhf et al., 20001). Although *domino* does not itself induce homeotic transformation,

it was shown that the lack of *domino* exhibited classical cell proliferation defect, and interestingly, enhanced Polycomb group mutations and counteracted trithorax group effects (Ruhf et al., 2001). Therefore, it appears that indeed chromatin regulation plays a significant role in cell viability and in blood cell homeostasis in *Drosophila*.

An alternative explanation to the lack of an apparent effect by only MLL fusion proteins on blood cell differentiation and/or proliferation resides with the ability of MLL fusion proteins to interact with conserved transcription factors required for blood cell development. In this case, MLL fusion proteins presumably cannot interact with transcription factors conserved between mammalian and *Drosophila*, such as Lozogen (AML1 homolog), Serpent (GATA homolog), glial cells missing, or hopscotch (JAK kinase), necessary for differentiation of hematopoietic lineages (Harrison et al., 1995; Lebstky et al., 2000). Section 1.5.2 alluded to the limited conservation between TRX and MLL at their N-termini. Thus, the lack of over-proliferating blood cells in *Drosophila* may be a consequence of this unconserved MLL N-terminal sequence, with the implication that MLL fusion proteins cannot directly interact with putative transcription factors that may require TRX for their proper activities. The generation of an N-terminal TRX with AF9 or with AF4 sequence would clarify if this speculation is indeed true, and whether over-proliferation of blood cells can then be detected as reported for MLL-AF9 expressing mice (Dobson et al., 1999).

An important point to consider is that mice harbouring the MLL-AF9 translocation were demonstrated to not develop leukaemia until a gestation period of 4-12 months, as in the human case (Corral et al., 1996). This study indicated that additional secondary hits are required for the onset of leukaemia. The fact that leukaemia was not observed in MLL fusion protein expressing flies supports the mouse data. Possibly, the emergence of secondary mutations, which generate leukaemia in *Drosophila*, is precluded by the MLL-AF9 and ML-AF4 induced lethality.

In addition to characterising the effect of MLL fusion proteins on hematopoeitic development, interference with apoptotic pathways was considered as a venue through which MLL fusion proteins may cause fly lethality. If indeed MLL fusion proteins are able to suppress or enhance apoptosis, as reported by studies based on mammalian cell culture (Section 1.5.4), it would be expected that tissue morphology would be altered and that

suppression or enhancement of apoptotic cells should be confined to cells expressing the MLL fusion proteins. As shown in Figure 3.8, neither of the two predictions existed. Therefore, at least in the *Drosophila* model system, apoptosis appears to not be one of the direct targets through which MLL fusion proteins interfere. The differing conclusion between the fly and mammalian cell culture data may reflect the behaviour of MLL fusion proteins in a relevant, developing, multi-cellular environment versus in transformed cell lines, which intrinsically possess altered cell cycle control points. Taken together, it appears that at least, MLL fusion proteins do not interfere with apoptosis as their primary target during leukemogenesis, although it cannot be excluded that MLL fusion proteins require components of a specific apoptotic pathway for their effect and for which is not present at the time the TUNEL assay was performed in *Drosophila*.

The finding that chromatin aberrations have been compromised in MLL-AF9 and in MLL-AF4 expressing transgenic flies offers an alternative route through which MLL fusion proteins target. In light that tumour inducing fly mutants and mutants deficient for hematopoetic components are linked with chromatin regulation, MLL-AF9 and MLL-AF4 may disrupt chromatin integrity as their primary function. Thus, the late manifestation of lethality by MLL-AF9 and by MLL-AF4 may reflect the time required for MLL fusion proteins to completely disrupt chromatin integrity either directly, or more likely, by modulating the activity of chromatin regulators.

CHAPTER 4

Chromatin effects observed in late lethal *trx* mutants and in MLL-AF9 transgenic fly lines are reminiscent of replication mutant phenotypes.

4.1 Introduction

Portions of the results presented in Chapter 3 alluded to an effect on the proper formation of higher order chromatin by MLL fusion proteins and suggested that polyploidy exists in both MLL and MLL-AF4, but not in MLL-AF9. Both these characteristics are shared by several tumour suppressor mutants in *Drosophila* (Riede, 1996b), and may reflect functions directly associated with specific MLL fusion proteins.

The polytene-like structures detected in the MLL-AF4 transgenic flies may reflect the deregulation of cell cycle control points, allowing continuous rounds of genomic replication. In *Drosophila*, a screen for late third instar larval lethals identified numerous mutants compromising the function of essential components regulating the cell cycle (Gatti and Baker, 1989). Interestingly, these so called "mitotic mutants" exhibit similar lethal phenotype as in MLL-AF9 and of MLL-AF4 transgenic flies. Although polyploid cells could be observed from squashed colchicine-treated brain preparations for some mitotic mutants, additional phenotypes, such as effects on chromatin condensation and on cell cycle progression, have been documented (Gatti and Baker, 1989). The brain squash preparation allows the detection for the presence of abnormal accumulation of mitotic figures from one nucleus (polyploidy), of aberrant chromatin condensation, and of improper cohesion between sister chromatids. Analysing colchicine-treated brain squashes would confirm if MLL and MLL-AF4 indeed have an influence on the cell cycle. If the polytene-like structures observed reflect cells that have undergone endoreduplication, metaphase spreads of affected cells should exhibit degrees of bundled sister chromosomes. In this respect, it is expected that polyploid cells should be observed for MLL and for MLL-AF4, but not in MLL-AF9.

A subtype of the mutants identified from the above screen mentioned (Gatti and Baker, 1989) has been reported to affect DNA replication and has been shown to play a role in cell cycle control checkpoint(s) (Krause et al., 2001). Mutants corresponding to different components of the replication machinery display aberrant sister chromatid cohesion, chromatin condensation and cell cycle progression defects, and compromised the timing of replication (Loupart et al., 2000; Pflumm and Botchan, 2001).

Two of the three mutants belong to the ORC (Origin Replication Complex) family, which act in a complex comprised of six subunits, designated ORC1-6. The ORC complex is thought to provide a "landing pad" for the pre-initiation complex prior to S phase, although yeast experiments indicate that the complex is not required for the activation of origins (Hua and Newport, 1998; Lee and Bell, 2000). The ORC subunits are in general bound to origins throughout the cell cycle, with the exception of ORC1 and ORC2 (Asano et al., 1999; Royzman et al., 1999). ORC2 binding is relieved starting at prophase, and only resumes at anaphase (Loupart et al., 2000). The recent characterisation of mutants for ORC2 and ORC5 reveals that late larval lethality is a result of inhibited cell proliferation due to a first arrest at S phase, as expected (Loupart et al., 2000; Pflumm and Botchan, 2001). The observation that in ORC2 and ORC5 mutants, a higher metaphase to anaphase ratio exists as compared to wild-type, indicated that defect in replication causes a second arrest point at metaphase. In addition, metaphase-like figures appeared highly condensed with interspersed decondensed chromatin fibers. Three different classes of chromosomal aberrations were documented for ORC2 and ORC5 mutants (Pflumm and Botchan, 2001). The most severe class of aberrant mitotic figures consisted of less condensed, shorter chromosomes that possibly form as a result of loss of sister chromatid cohesion, or of precocious sister chromatid separation from metaphase to anaphase transition. This conclusion is supported by a mutant of "proliferation disrupter", a centromeric binding protein, which displays similar mitotic figures in metaphase spreads as a consequence of defect in sister chromatid cohesion (Török et al., 1997). In addition, it was noted that the phenotype for chromosomal aberrations in ORC2 and ORC5 mutants became progressively more severe in older larvae (Loupart et al., 2000; Pflumm and Botchan, 2001). Presumably, the severity of the phenotype is correlated with the amount of maternal ORC contribution. The maternal pools for replication or cell cycle proteins deplete over time, and consequently, manifestation of lethality occurs late in development. Speculatively, the defects in mitotic figures may be a general phenotype of replication defective mutants since PCNA (proliferating cell nuclear antigen) and MCM4 (mini-chromosome maintenance 4) (factors required during DNA replication) mutants also exhibit the same aberrations (Pflumm and Botchan, 2001).

The recent characterisation of another replication protein, Rfc4 (Replication Factor C), linked chromosomal and replication defects with cell cycle checkpoints (Krause et al., 2001). Assuming that Rfc4 only functions in DNA replication, it would be expected that cells lacking Rfc4 should arrest at S phase and not enter mitosis. The report of a higher mitotic index to replication activity in Rfc4 mutants as compared with wild-type suggested that additional functions are mediated by Rfc4 during the cell cycle after S phase (Krause et al., 2001). Indeed, treating *Rfc4* mutants with several agents, that damage DNA and lead to cell cycle arrest, revealed that cell cycle checkpoints were defective in *Rfc4* mutants since a significant fraction of treated cells progressed through mitosis (Krause et al., 2001).

The described molecular characterisations of *orc2*, *orc5*, and *Rfc4* mutants point to a much more intimate connection between DNA replication and cell cycle progression, and may, in fact, be critical targets during cell transformation. Chapter 4 begins by presenting data, which link TRX mediated chromatin integrity at late developmental stages with DNA replication. The results demonstrate that TRX has additional, distinct functions other than its maintenance role in embryogenesis, and imply that chromatin integrity is an important step for the allowance of DNA replication. Only MLL-AF9 was observed to display similar chromatin defects in diploid cells as late lethal *trx* mutants and replication defective mutants, although a hyper-initiation of DNA replication was observed. MLL and MLL-AF4 expressing diploid cells were found to exhibit wild-type mitotic figures and were associated with normal DNA replication. Taken together with the earlier finding that the polytene architecture from salivary glands of MLL-AF4 and MLL-AF9 (Chapter 3) has been altered, accumulative evidence implies that the extent chromatin integrity is compromised, varies among MLL fusion proteins. Moreover, these results indicate that MLL fusion proteins possess numerous and distinct functions aimed at deregulating various pathways controlling the cell cycle. The observed replication effect by MLL-AF9 and not by MLL-AF4, suggests that the C-terminal AF9 sequences may aberrantly recruit pre-initiation complexes to origins. The results presented in Chapter 4 will be discussed in relation to models proposed for replication defective mutants and will consider the novel functional role for TRX at late developmental stages.

4.2 Results

4.2.1 *trx* mutants, lethal at late larval to pupal stages, exhibit decondensed chromatin aberrations and reduced DNA replication.

Data from an extensive study on various alleles harbouring different *trx* mutations suggested that TRX is able to incorporate into several complexes whose functions are required at different times in development (Breen, 1999). In a heterozygous background, all *trx* alleles are viable, indicating that half the dosage of wild-type TRX is sufficient for proper fly development. On the other hand, lethality associated with homozygous *trx* mutants range in its severity depending on the allele. The null TRX mutant, trx^{b11}, is embryonic lethal, whereas several other mutations are lethal at larval or at pupal stages. Two mutants with interesting characteristics include the JY16 and the E3 alleles. The trx^{JY16} mutant is larval lethal when combined with a null *trx* allele (Breen, 1999). Interestingly, this allele was found to consist of a translocation point that presumably transcribes an active fusion protein of the longer TRX II isoform, although direct evidence is lacking (Breen, 1999). trx^{E3} corresponds to a deletion upstream of the PHD fingers and was observed to exhibit growth defect phenotypes similar to signal transduction mutations (Breen, 1999). It is interesting to speculate why minor deletions of the *trx* sequence result in late larval or pupal lethality. A possible explanation may reflect additional function(s) for the TRX protein required for the proper maintenance of chromatin integrity at late development of the fly. In this case, the maternal contribution of TRX would allow development at embryogenesis. Hypomorphic TRX proteins, harbouring minor mutations of its sequence, may still acquire their ability to maintain the pattern of gene expression set-forth during embryogenesis, and allow development to proceed to later developmental stages, in contrast to the null *trx* mutant which cannot survive past embryogenesis. The lack of a presumably distinct and vital TRX function required for late development would result in the manifested late lethality. To address this hypothesis, dissection of larvae harbouring a copy of the null *trx* allele (trx^{b11}) and a copy of the deleted *trx*

sequence (trx^{JY16} or trx^{E3}), was performed to look for any abnormalities of the imaginal discs giving rise to the adult fly.

As shown in Figure 4.1, the brains from larval or pupal *trx* mutants were observed to be significantly smaller in size than wild-type. This suggests that a lack of cell proliferation or an elevation of apoptosis may be the cause for the retarded brain growth. By analysing isolated brains for apoptosis with the TUNEL assay (see Chapter 3), it was observed that apoptosis was apparently not altered in *trx* double mutants (Figure 4.8). Interestingly, isolated brains from DNA replication defective mutants have been shown to exhibit similar brain-size and brain morphological phenotypes as *trx* mutants (Krause et al., 2001; Pflumm and Botchan, 2001). To investigate if lack of DNA replication may be a cause for the apparent inhibited cell proliferation, isolated *trx* mutant brains were incubated in a BrdU solution for 1.5 hours at 25 °C. Staining with a specific monoclonal antibody against BrdU revealed that late larval or pupal *trx* mutants displayed a strikingly reduced level of replicating cells as compared to wild-type (Figure 4.1).

A collection of mutagenised *Drosophila* stocks, which are larval to pupal lethal, comprise of the mitotic or replicative defective mutants (see Section 4.1). Both classes of mutants often share similar variations of chromosomal aberrations and exhibit cell cycle checkpoint or progression defects. In light of the recent discovery that *trx* mutants are associated with a lack of proliferating cells, it is of great interest to document the state of chromatin integrity in these affected cells. For this, the *Drosophila* brain has been traditionally employed as the tissue of preference for studies that aim to look at defects in chromosomal structures or in cell cycle progression. The brain squashing technique used to present the following results, was initially established by Maurizio Gatti and members of his laboratory (see Section 7.2.9), and allows the visualisation of mitotic figures, reaching their most condensed structure at metaphase.

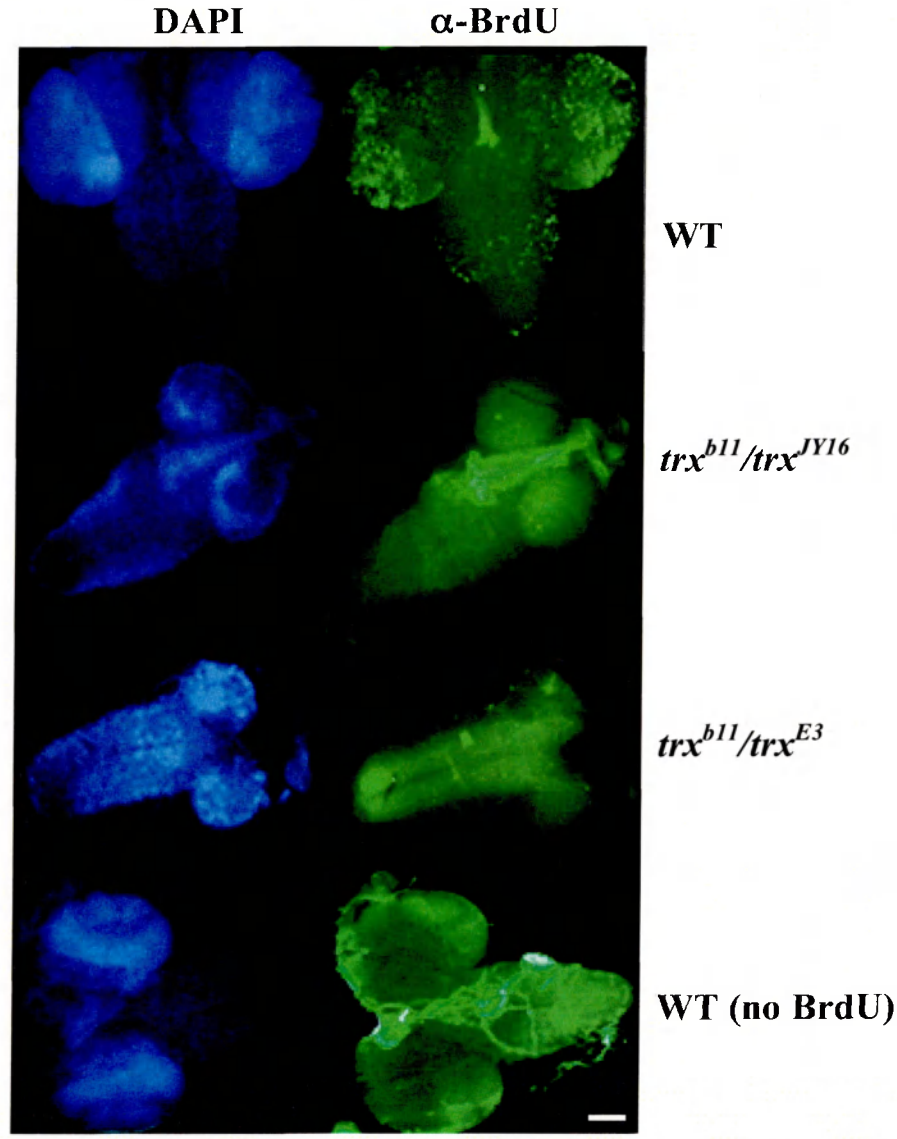


Figure 4.1. Late lethal *trx* mutants are associated with DNA replication defect. Isolated brains were incubated in 0.1 mg/ml BrdU solution at 25 °C for 1.5 hours, and subsequently, processed for whole-mount staining with monoclonal BrdU antibodies. Wild-type brains, incubated in BrdU solution, show a characteristic pattern of replicating cells, confined to the lobes and to the mid-section of the brains. Wild-type brains without BrdU incubation are not stained. trx^{b11}/trx^{JY16} and trx^{b11}/trx^{E3} mutant brains do not display any nuclear BrdU staining, indicating that cells are not replicating. Also, note the underdeveloped brain lobes isolated from *trx* mutants as compared to wild-type. Bar = 100 μm.

Brain squashes were performed with isolated mutant *trx* brains, and DAPI stained chromatin was analysed for chromatin aberrations. As depicted in Figure 4.2, four chromosomes comprise the *Drosophila* genome as indicated. Two pairs with long chromosomal arms correspond to the II and III chromosomes. A V-like structure describes the X chromosome of *Drosophila*, while the Y-chromosome consists of one dense tubular structure. The final pair of chromosome IV in *Drosophila* is composed of mostly heterochromatin and appears as two dots in metaphase spreads.

Figure 4.2 presents the chromatin phenotype associated with the appropriate *trx* mutant genotypes. In both the trx^{JY16} and trx^{E3}, chromatin decondensation was observed, resembling "prophase-like" chromatin. Remarkably, the lack of distinct metaphase spreads, such as those for wild-type, suggest that nuclei lacking TRX function cannot progress past S phase of the cell cycle, by possibly not generating proper condensed chromosomes required for completion of the cell cycle. Treatment of *trx* mutant brains with colchicine, which arrests cells at metaphase, revealed an increase of "normal" metaphase spreads to 10-20 per brain. This number contrasts drastically with colchicine treated wild-type brains, which exhibited 100-150 metaphase spreads per brain. Again, this result emphasizes the inability of cells lacking *trx* to complete the cell cycle.

The analyses with late lethal *trx* mutants connect for the first time replicating cells with the state of TRX mediated chromatin integrity. The striking result that virtually no metaphase spreads from whole *trx* mutant brains exist, accounts for the lack of proliferating cells and for the retardation of cell growth as shown by their inherent smaller brains. In addition, the lobes where the majority of the proliferating cells reside appear to be underdeveloped as compared to wild-type (Fig 4.1, compare wild-type with the *trx* mutants). The presence of an altered chromatin structure in trx^{JY16} and trx^{E3} mutants is well linked with the replication defect identified. These results imply that TRX possess an additional function, which is necessary at late development and which is distinct from its earlier maintenance role, for completion of late development.

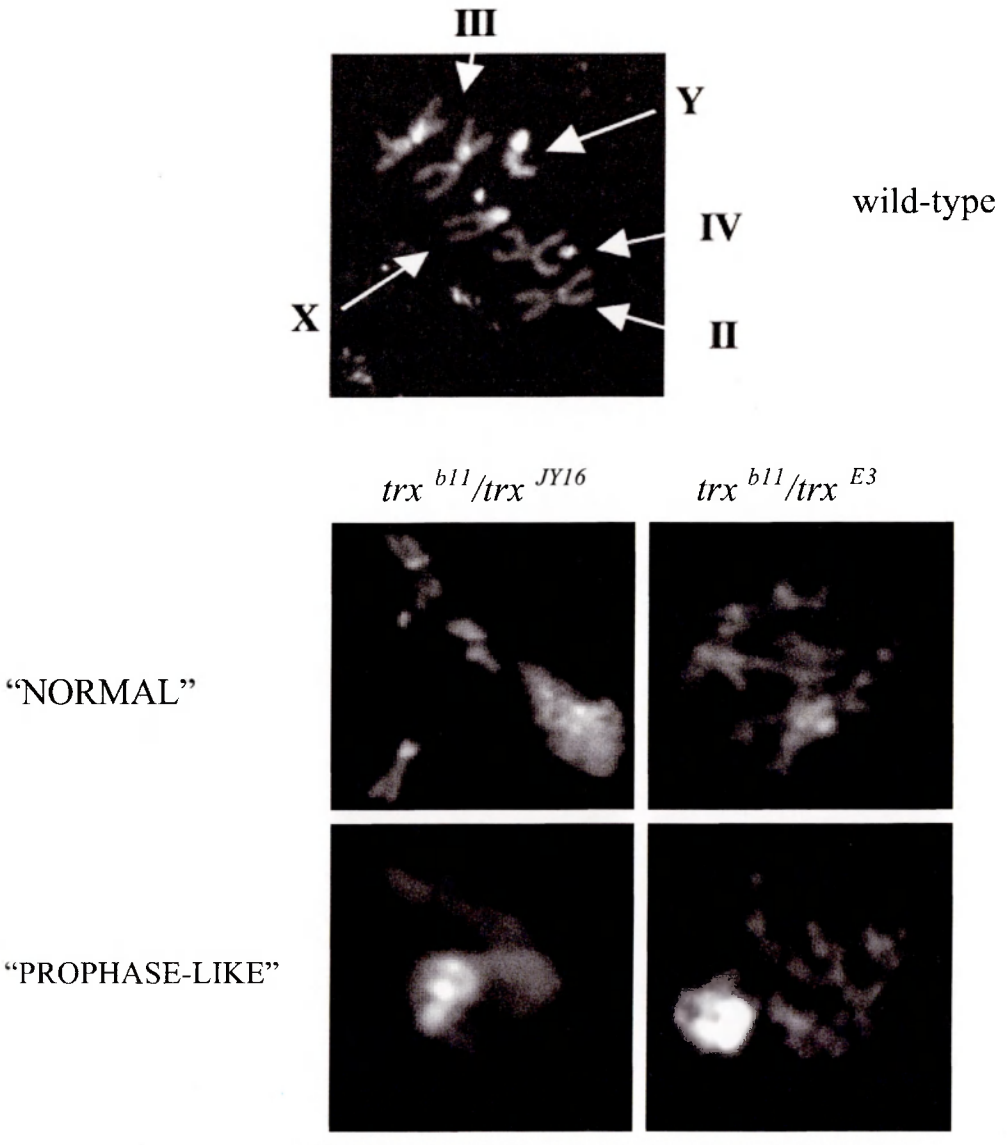


Figure 4.2. Abnormal structures in metaphase spreads from brains of *trx* mutants. Flies harbouring either the trx^{b11}/trx^{JY16} or the trx^{b11}/trx^{E3} genotype, are able to develop to larval or pupal stages, respectively. Brain squashes were performed for these rare surviving *trx* mutant larvae. It was observed that a lower number of cells, as detected by DAPI staining were present in one squashed brain as compared to wild-type. Most of the cells appear to be in interphase as characterised by dense, small nuclei. The remaining nuclear spreads appear "prophase-like", resembling an initial phase of chromosomal condensation. Around +/- 50% of *trx* mutant cells display this particular nuclear structure. Only 1 or 2 "normal" metaphase chromosomes, structurally resembling wild-type metaphase mitotic figures, were detected per *trx* mutant brain (suggesting that in general cells do not reach metaphase). The chromosomes, however, seem fluffy. On the other hand, wild-type metaphase spreads show homogeneously condensed mitotic figures, as indicated.

4.2.2 Chromatin defects observed in MLL-AF9 but not in MLL-AF4 transgenic fly lines.

The discovery that a lack of a proper TRX function has consequences in cell proliferation, support well published results linking MLL with cell cycle regulation (Cui et al., 1998). It was shown above that mutated versions (deletion, translocation) of the *trx* sequence allows development past its normal embryonic lethal stage (in *trx* -/-), and appears to alter chromatin structures required for proper cell proliferation. In this respect, a mode of action by aberrant MLL proteins associated with leukaemia may be the impairment of MLL function similar to those in late lethal *trx* alleles. Specifically, the presence of MLL fusion proteins may interfere with the formation of chromatin structures mediated by TRX (and analogously in mammals, MLL) and required for properly controlled cell division. The generation of a relevant *Drosophila* model system to study the function associated directly with MLL leukaemic fusion proteins (Chapter 2) points to MLL-AF9 and MLL-AF4 inhibiting late *Drosophila* development. It is likely that MLL-AF9 and MLL-AF4 may prevent late TRX function necessary for appropriate cell proliferation, resulting in larval or pupal lethality, reminiscent of phenotypes in the late lethal *trx* mutants. To explore this possibility, analyses of the chromatin configuration associated with MLL-AF9 and MLL-AF4 expressing flies were performed.

MLL, MLL-AF9, and MLL-AF4 transgenic flies were crossed to an ubiquitous Gal4 driver (daughterless Gal4, which is capable of inducing a high protein level), and progeny were monitored to be lethal at 29 °C (Chapter 2, Fig 2.6). Figure 4.3 presents chromosomal metaphase spreads from wild-type, MLL, MLL-AF4, and MLL-AF9 transgenic fly lines. Spreads from MLL and MLL-AF4 transgenic brains show that MLL mitotic figures are indistinguishable from wild-type. However, metaphase spreads from MLL-AF9 transgenic brains show a striking effect on chromatin structure. Mitotic figures from MLL-AF9 were characterised by decondensed portions along the chromosomal arms of the mitotic figures (Fig. 4.3d, arrows). These structures were classified as Class II. Interestingly, these chromosomal defects have been reported to exist for replication defective mutants (Pflumm and Botchan, 2001), and appear to be less severe in relation to the decondensed effects observed in late *trx*

mutants (Fig 4.1). As shown in Figure 4.3E, a large portion of cells possessed fragmented chromosomes (designated as Class III). Like for Class II chromosomal defects, Class III-type chromosomal figures have been observed in replication defective mutants (Loupart et al., 2001; Pflumm and Botchan, 2001). Careful analyses have revealed that although not all cells expressing MLL-AF9 displayed an observable aberrant chromosomal structure (Class I), a significant percentage of Class II and Class III metaphase spreads were associated with the presence of MLL-AF9 (Fig. 4.4).

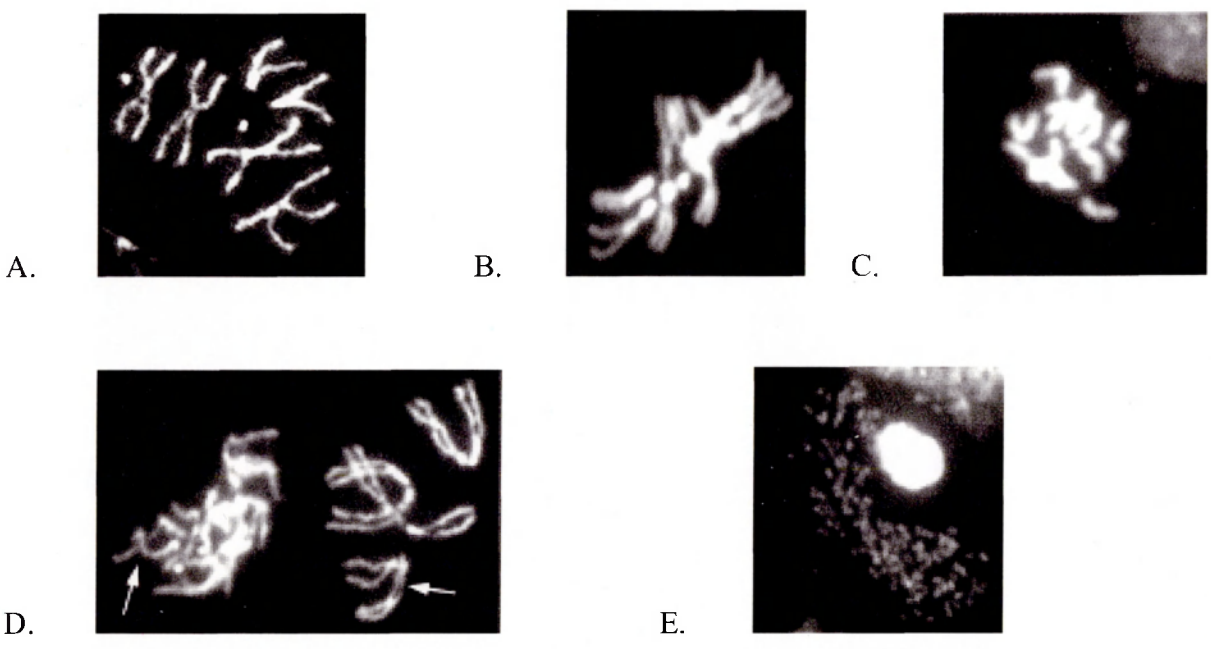


Figure 4.3. MLL-AF9, but not MLL or MLL-AF4, affects global chromatin structure. (A) A wild-type metaphase spread of wild-type brain cell is associated with homogeneous condensation of chromatin into chromosome arms. (B) Metaphase spreads from MLL (B) and from MLL-AF4 (C) expressing larval brain cells display the same chromatin conformation as in wild-type (A). (D) Aberrations in chromosome structures were clearly observed in MLL-AF9 larval brains. Note the decondensation of chromatin along the arms of the mitotic figures (arrows). Such structures are absent in (A-C). (D) "Fragmented" mitotic spreads are frequently observed in MLL-AF9 transgenes. (All figures were captured at 100x magnification using a Leica Microscope.)

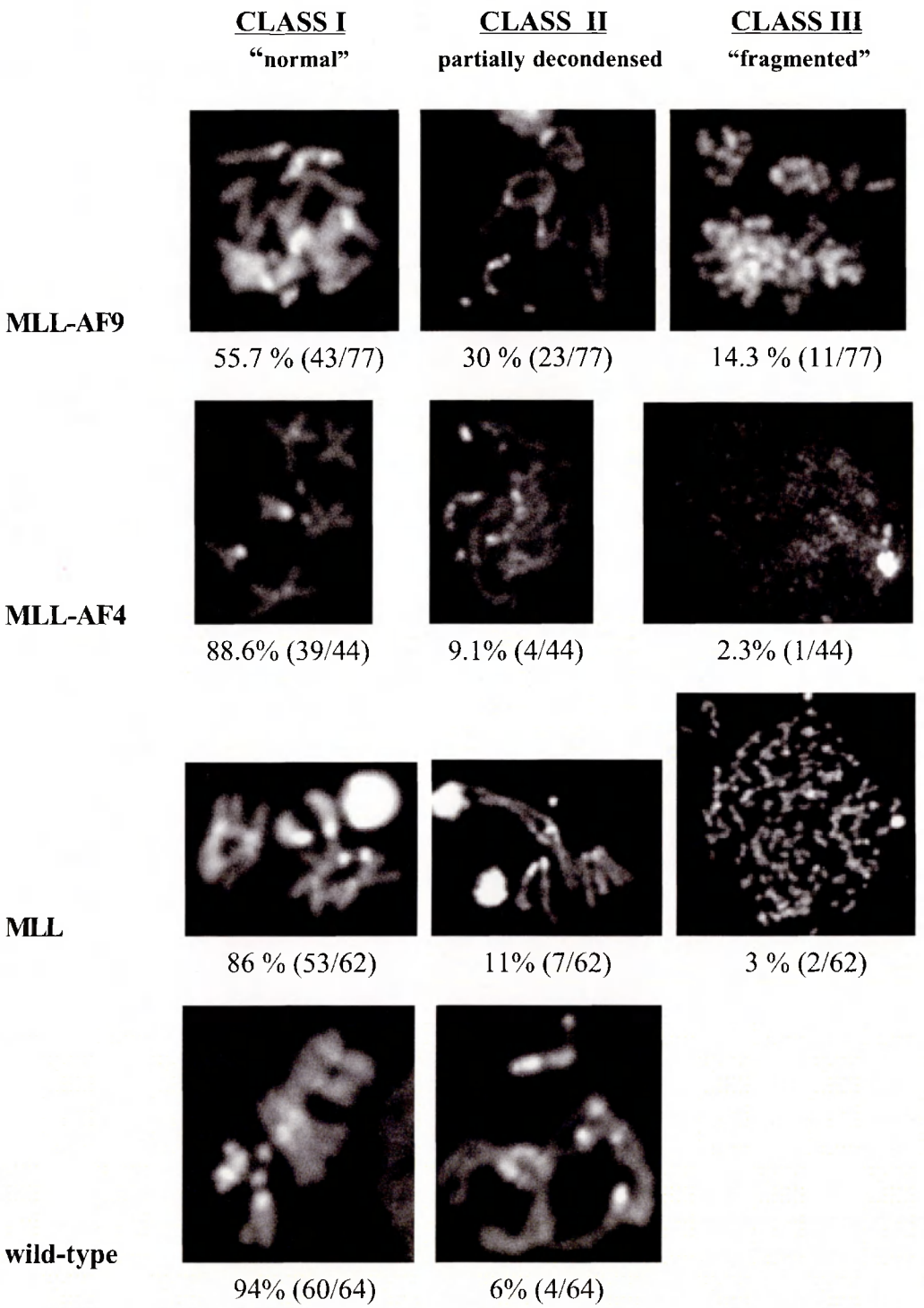


Figure 4.4. Detailed analyses reveal that MLL-AF9 significantly alters chromatin condensation. At least four independent sets of experiments were performed to document in more detail the chromatin condensation defects associated with MLL-AF9 expressing larvae. Three types of metaphase spreads were noted. "CLASS I" refers to metaphase spreads resembling wild-type mitotic figures (see Fig 4.3A). "CLASS II" refers to moderately modified chromatin structure (see Fig 4.3D). "CLASS III" displays mitotic figures that appear to have been fragmented (see Fig 4.3E). As shown above, altered chromatin structures were not significantly observed in MLL or in MLL-AF4 expressing larvae. Percentages indicate the frequency by which the distinct metaphase spreads were observed in two brains. (magnification: 100x)

Interestingly, a lower metaphase to anaphase ratio, for MLL-AF9, and minimal for MLL-AF4 expressing flies, as compared to MLL and to wild-type was detected (Fig 4.5A). Replication deficient mutants have been reported to possess a high metaphase to anaphase ratio as compared to wild-type (Loupart et al., 2000; Pflumm and Botchan, 2001), indicating that a second arrest point has occurred at metaphase. The fact that the ratio is lower in MLL-AF9 may reflect MLL-AF9's ability to allow continued cell cycle divisions without proper checkpoints, or alternatively, to increase the rate of replication in MLL-AF9 cells, resulting in greater numbers of observable anaphase. The finding that MLL-AF9 and MLL-AF4 were associated with a slight increase in mitotic index relative to MLL, supports their ability to allow cell cycle progression (Fig. 4.5B,C).

Taken together, results indicate that MLL-AF9 may cause larval to pupal lethality by possibly altering chromatin structures which are required for DNA replication and which are likely, mediated by TRX's later function in development. If this scenario is proven to be true, the presence of more condensed chromosomal structures as compared to those detected in late lethal *trx* mutants, may reflect MLL-AF9's inability to completely inhibit the formation of DNA replication structures mediated by TRX. Alternatively, additional functions associated with MLL-AF9 may contribute to the manifestation of the lethal phenotype. In this respect, the facts that MLL-AF4 exhibited relatively normal mitotic figures and slightly affected cell cycle progression as monitored by the mitotic index, suggest that different MLL fusion proteins affect late TRX function at different levels, and most likely, require additional functions to abrogate cell cycle control.

A.

Fly Lines	Metaphase	Anaphase	Metaphase: Anaphase Ratio	Relative to WT
wild-type	41	19	2.16	1
MLL	29	14	2.07	0.96
MLL-AF4	24	15	1.60	0.74
MLL-AF9	18	25	0.72	0.33

B.

Fly Lines	Phospho-H3	Total No. Cells	Mitotic Index	Relative to MLL
MLL	23	1344	0.0049	1
MLL-AF4	29	1120	0.007	1.4
MLL-AF9	50	1100	0.0026	1.9

C.

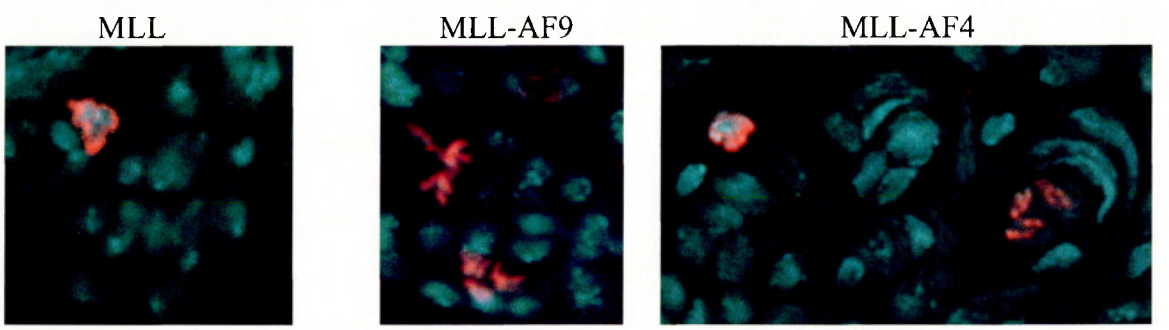


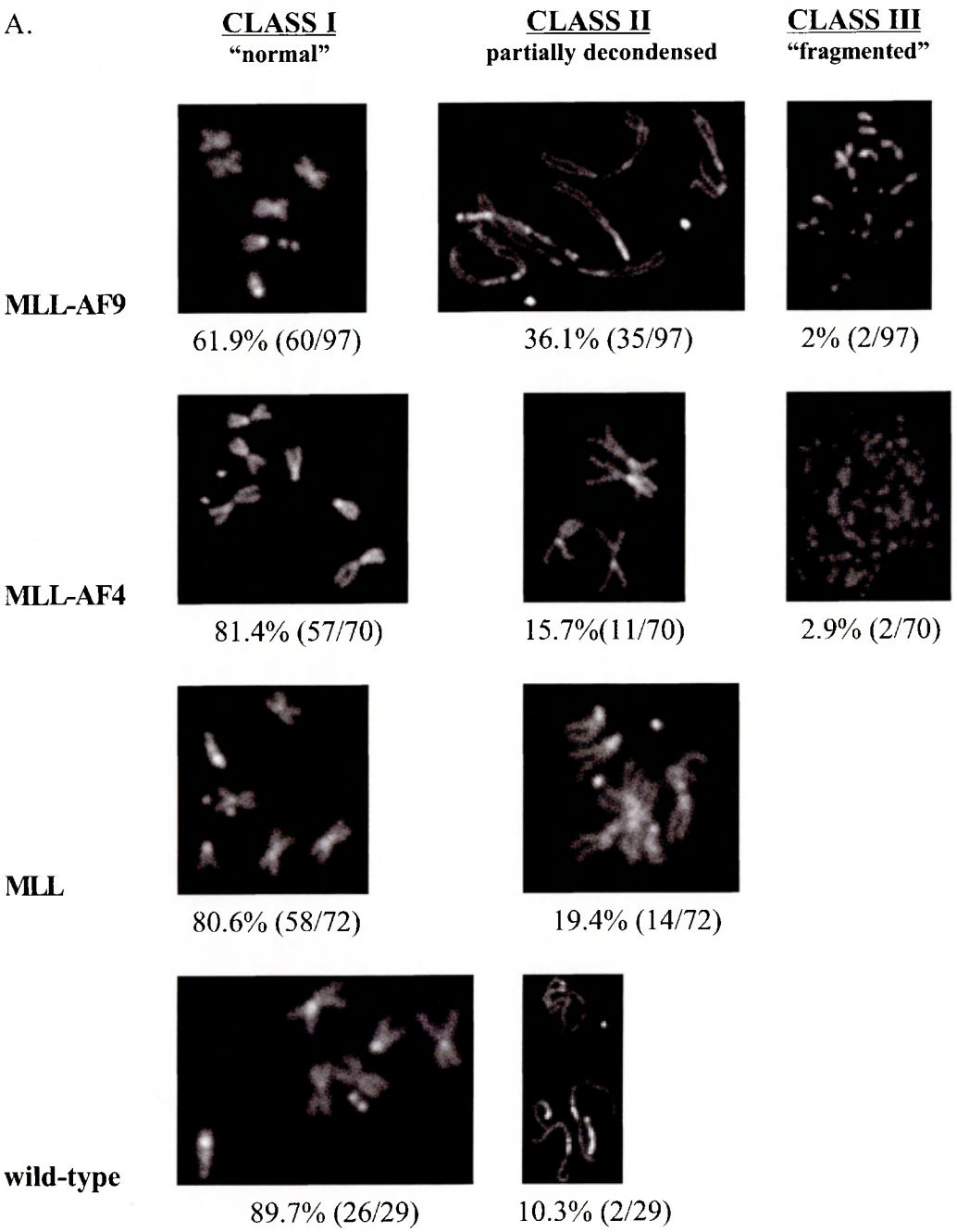
Figure 4.5. Metaphase to anaphase ratio and mitotic index suggest that MLL-AF9 cells proliferate faster than MLL and MLL-AF4. (A) The number of cells in metaphase and anaphase was counted for two brains in three different experimental set-ups. The ratio presented is relative to wild-type. The low ratio associated with MLL-AF9 may reflect the capability of MLL-AF9 to allow cell cycles to progress at a quicker rate. (B) Phospho-H3 marks cells that have entered the cell cycle. A slight effect on the mitotic index by MLL-AF9 and MLL-AF4 indicates that MLL fusion proteins allow cell cycle progression. (C) Controls demonstrating that the Phospho-H3 staining is relevant. As shown, the antibodies detect cells going through the cell cycle (Red: PhosphoH3, Blue: DAPI).

4.2.3 Colchicine and hypotonic treatments of metaphase spreads reveal that polyploidy is non-existent.

The presence of polytene-like structures observed in MLL and in MLL-AF4 transgenic lines suggested that polyploid cells exist. To confirm if cells in MLL and MLL-AF4 transgenic lines are polyploid, isolated brains were treated with colchicine for 1.5 hours at 25 °C, followed by a hypotonic treatment. Treatment of MLL-AF9 expressing tissues with colchicine presumably should not reveal any polyploid cells, since polytene-like structures were not detected (Chapter 3).

The treatment of colchicine, which prevents microtubule polymerization and thus induces disappearance of mitotic spindles, causes cell cycle arrest at metaphase, whereas hypotonic treatment swells the cells, allowing sister chromatids to spread. Figure 4.6A illustrates colchicine-treated metaphase spreads from appropriate fly lines. As shown, polyploidy was not observed in metaphase spreads from all transgenic lines. Although metaphase spreads resembling chromosomal aberration Class III appear to be polyploid, the numbers of observable spreads were minimal for MLL-AF9 and MLL-AF4 transgenic lines compared to MLL transgenic flies and to wild-type. It should be noted that in several spreads loss of chromosomes was detectable (Figure 4.6B).

A.


CLASS I
"normal"
CLASS II
partially decondensed
CLASS III
"fragmented"
MLL-AF9
61.9% (60/97)
36.1% (35/97)
2% (2/97)
MLL-AF4
81.4% (57/70)
15.7%(11/70)
2.9% (2/70)
MLL
80.6% (58/72)
19.4% (14/72)
wild-type
89.7% (26/29)
10.3% (2/29)

B.

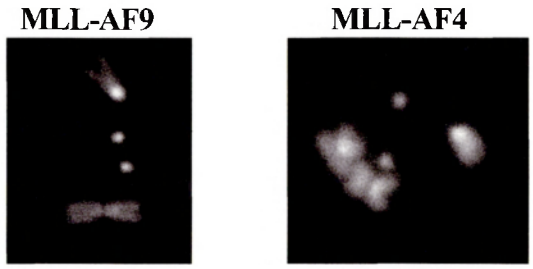


Figure 4.6. Colchicine-treated brain squashes reveal that polyploidy is non-existent in MLL and MLL fusion transgenic flies. Isolated brains from appropriate transgenic lines were treated with colchicine for 1.5 hours at 25 °C. Illustrated are metaphase spreads observed from these squashed brains. (A) Analyses of metaphase spreads were performed according to the three classes identified in Fig. 4.4. As shown, MLL-AF9 displays an appreciable percentage of Class II and Class III metaphase spreads as compared to wild-type and to MLL and MLL-AF4 expressing cells. (B) Intriguily, a significant number of metaphase spreads, which lacked chromosomes and which resembled Class I mitotic figures, were observed in MLL-AF9 and MLL-AF4 expressing fly lines. Percentages indicate the frequency by which the distinct metaphase spreads were observed in two brains.

4.2.4 *in vitro* BrdU incorporation reveals a hyper-initiation of DNA replication for MLL-AF9 transgenic flies

The discovery of chromosomal aberrations similar to those in replication defective and late lethal *trx* mutants encouraged experiments directed at exploring whether DNA replication was intact in MLL fusion transgenic lines. In light of the chromatin phenotype documented above, it is expected that only MLL-AF9 should associate with a defect in DNA replication.

The indication that abnormal DNA replication existed in MLL-AF9 came from experiments using BrdU as a marker for cells undergoing replication. Figure 4.7 depicts brains that have been labelled with BrdU for 1.5 hours *in vitro*. As shown, replicating cells are normally confined to the two optic lobes of the hemisphere and to the rental ganglion in wild-type *Drosophila*. This typical pattern of proliferation zones was observed in MLL and in MLL-AF4 transgenic flies. However, an increase of replicative cells was observed for MLL-AF9 transgenic flies. The effect on cell replication was tightly linked to the amount of protein levels since only the most severe, lethal transgenic line displayed, at this resolution, a higher

level of replicating cells under the same experimental condition (compare MLL-AF9-T2 (100% pupal lethality) vs. MLL-AF9-T5 (75% pupal lethal)).

The results presented here also correlate with the presence of the chromatin phenotype described above in MLL-AF9 and not in MLL nor in MLL-AF4 expressing cells. Interestingly, although chromatin aberrations were found to be similar to the published phenotype exhibited by replication defective *Drosophila* mutants (Loupart et al., 2000; Pflumm and Botchan, 2001) and to late lethal *trx* mutants (compare Fig 4.2 and Fig 4.3D and Fig. 4.4 MLL-AF9 Class II and Class III), the higher number of replicating cells observed for MLL-AF9 opposes the lack of replication in replication deficient and in late lethal *trx* mutants. This observation may indicate that MLL-AF9 may not only inhibit the late TRX function identified, but may also contribute dominant effects such as hyper-initiation of DNA replication. Alternatively, a delay in progression at S phase by MLL-AF9 would also explain the observed pattern of BrdU positive cells. In both respects, it appears that MLL-AF9 targets more than one pathway to disrupt controlled cell proliferation during leukemogenesis.

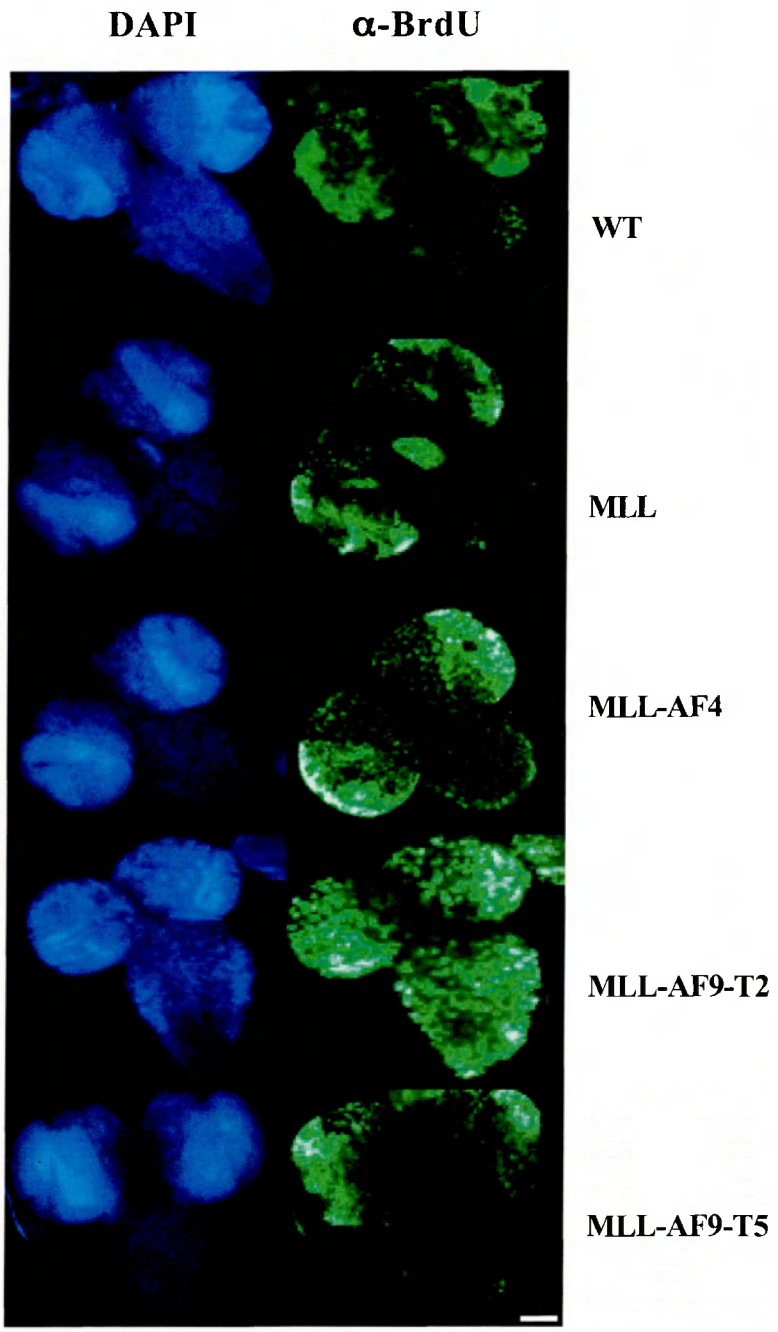


Figure 4.7. *in vitro* BrdU incorporation demonstrates that only MLL-AF9 affects DNA replication. Isolated brains from appropriate flies were subjected to *in vitro* BrdU treatment, and were subsequently stained for BrdU incorporation. As shown, nuclear staining of BrdU positive cells were equal for wild-type, MLL, and MLL-AF4. However, in MLL-AF9, a higher number of cells incorported BrdU indicating that initiation of DNA replication was elevated. This effect was dependent on MLL-AF9 levels since the only more severe transgenic line exhibited a clear defect (MLL-AF9-T2). Bar = 100 μm.

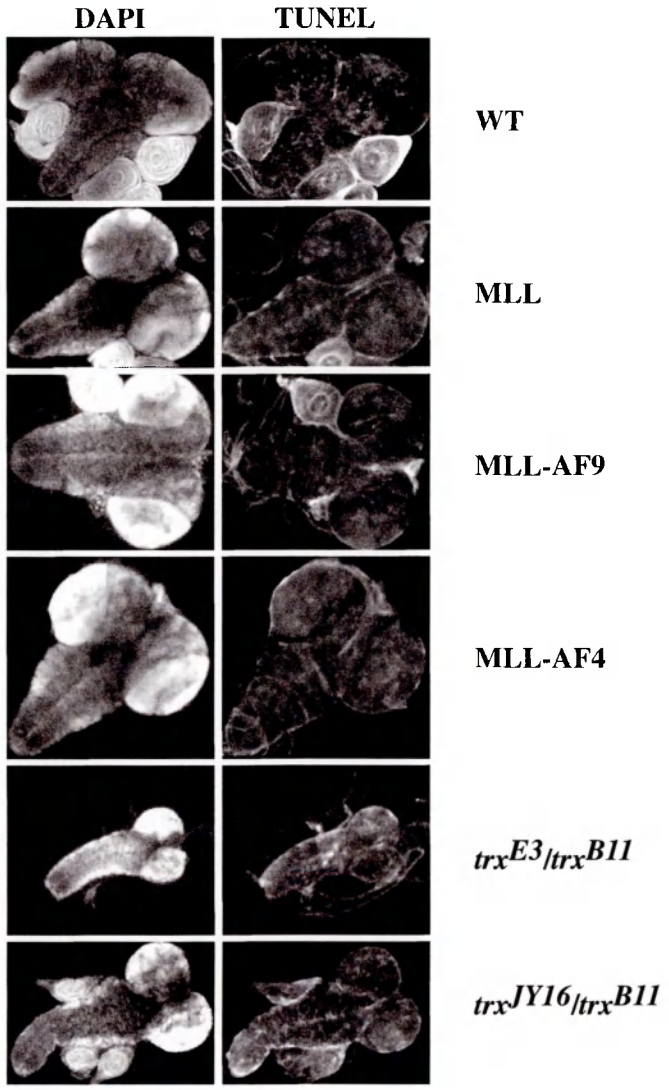
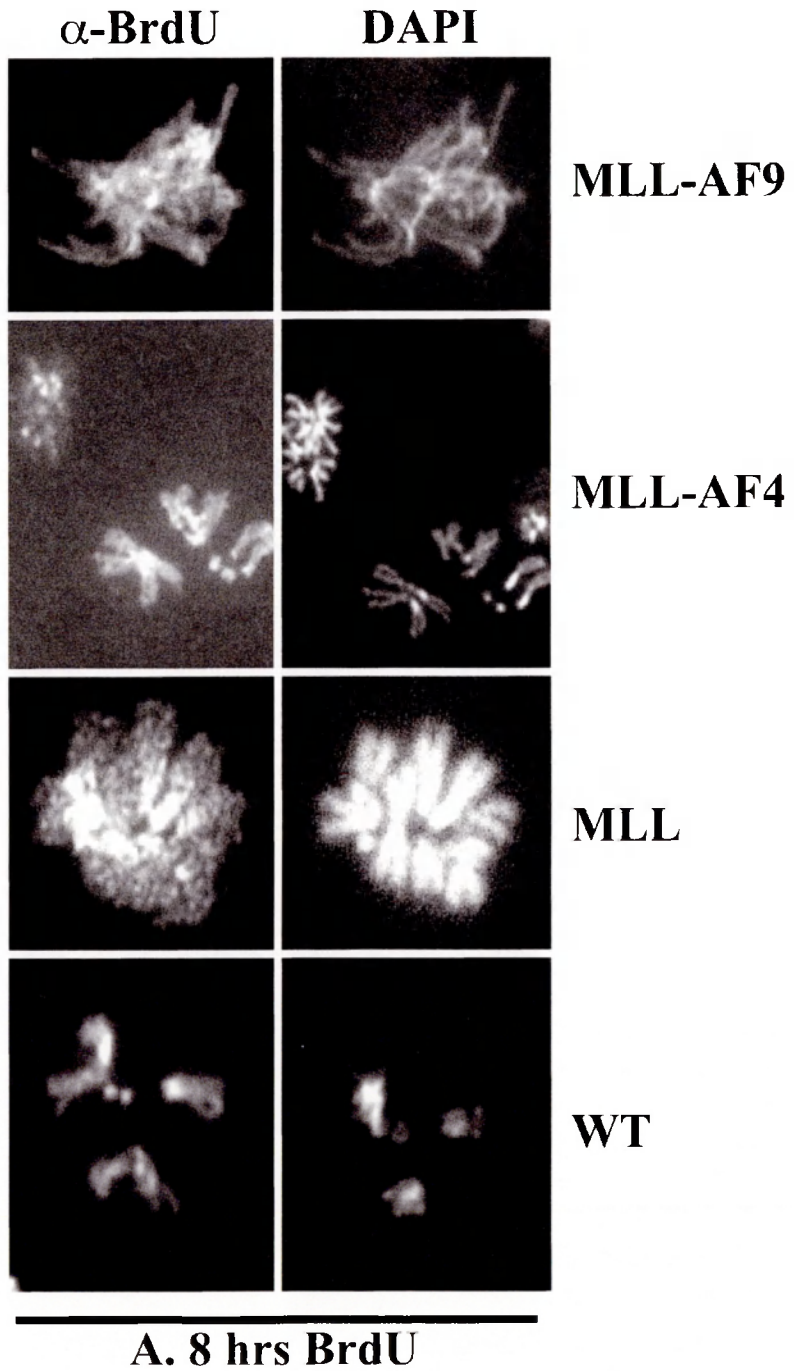
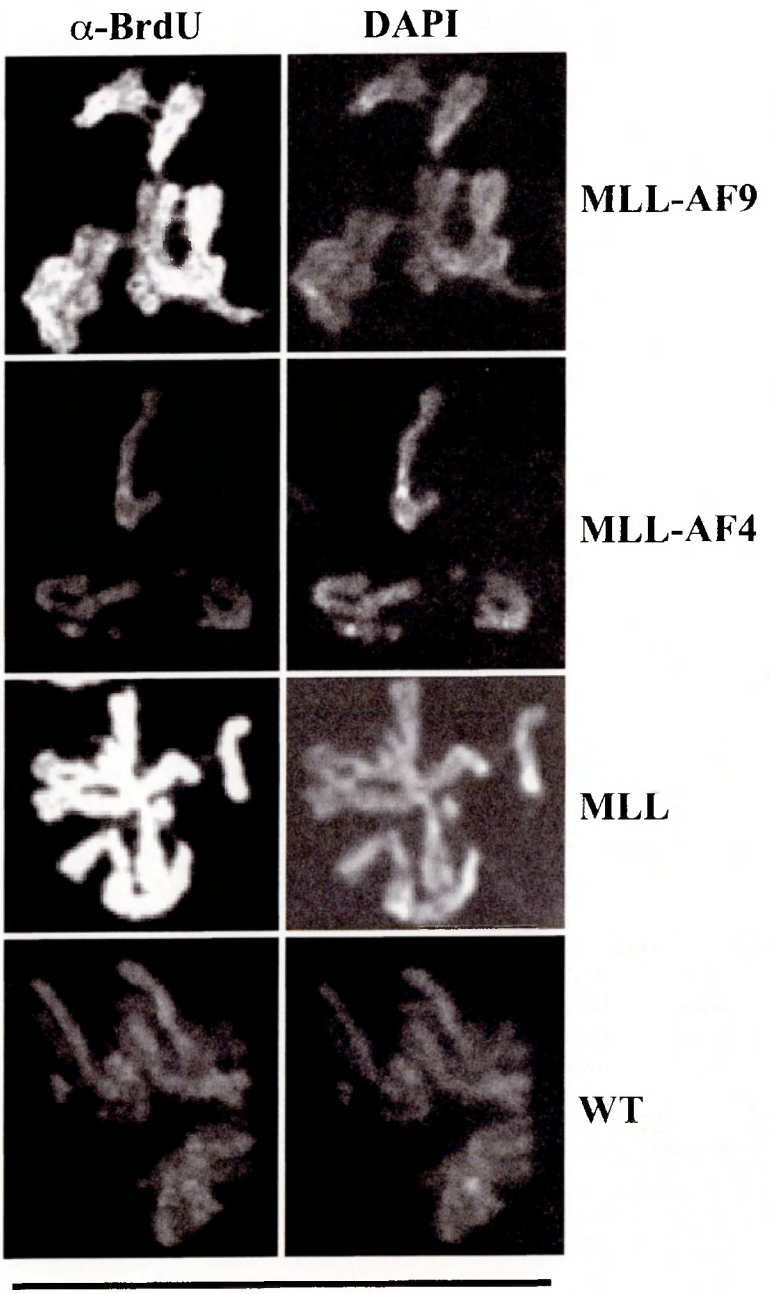


Figure 4.8. TUNEL assay on isolated brains from MLL and MLL fusion transgenic flies, and double *trx* mutants, reveals phenotypically similar apoptosis pattern as in wild-type brains. MLL, MLL-AF9, and MLL-AF4 were crossed to daughterless-Gal4 and raised at 29 °C. The *trx* double mutants were raised at 21° C. As shown above for one brain, isolated brains from wild-type 3rd instar wandering larvae raised at 29° C, display minimal apoptosis activity as detected by the TUNEL assay. Likewise, brains from all transgenic fly lines and *trx* double mutants exhibited similar phenotypes, indicating that an abnormal level of apoptosis is not present.

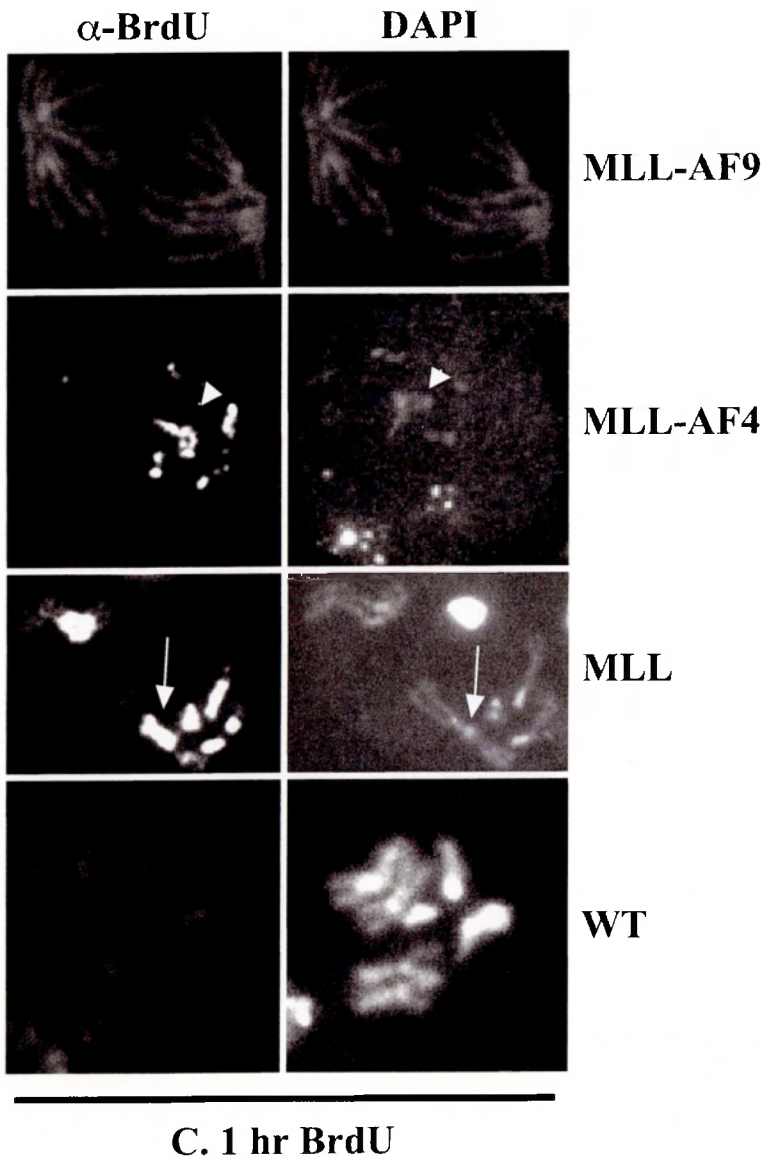
4.2.5 *in vivo* BrdU labelling indicates that cell cycle progression is perturbed in MLL-AF9 expressing flies.

It has been proposed that defect in the timing of DNA replication can lead to chromosomal aberrations (Loupart et al., 2000). To analyse in more detail the connection between replicating cells and chromatin structure defect, BrdU was fed to appropriate larvae for 20 minutes, 1 hour, 2-4 hours and for 8 hours. Feeding of larvae for 6-24 hours with BrdU is sufficient to observe complete BrdU labelling of mitotic figures in wild-type, while less than 4 hours BrdU *in vivo* labelling allows partial incorporation of BrdU into wild-type mitotic figures (Loupart et al., 2000). Figure 4.9 illustrates metaphase spreads obtained from various time points of BrdU exposure. At 8 hours, like wild-type, MLL, MLL-AF9, and MLL-AF4 mitotic figures were completely labelled with BrdU in a substantial number of cells (Fig 4.9A). At 2-4 hours BrdU labelling, whole or partial BrdU incorporated mitotic figures were observed for all MLL transgenic lines, although only rarely for wild-type (Fig. 4.9B). However, when pulsed with BrdU for shorter time intervals, it was observed that MLL-AF9 exhibited metaphase spreads that have partially incorporated BrdU, while mitotic figures from MLL, MLL-AF4, and wild-type were not observed to be BrdU positive (Fig. 4.9D).


α-BrdU
DAPI
MLL-AF9
MLL-AF4
MLL
WT
A. 8 hrs BrdU



B. 2-4 hr BrdU



C. 1 hr BrdU

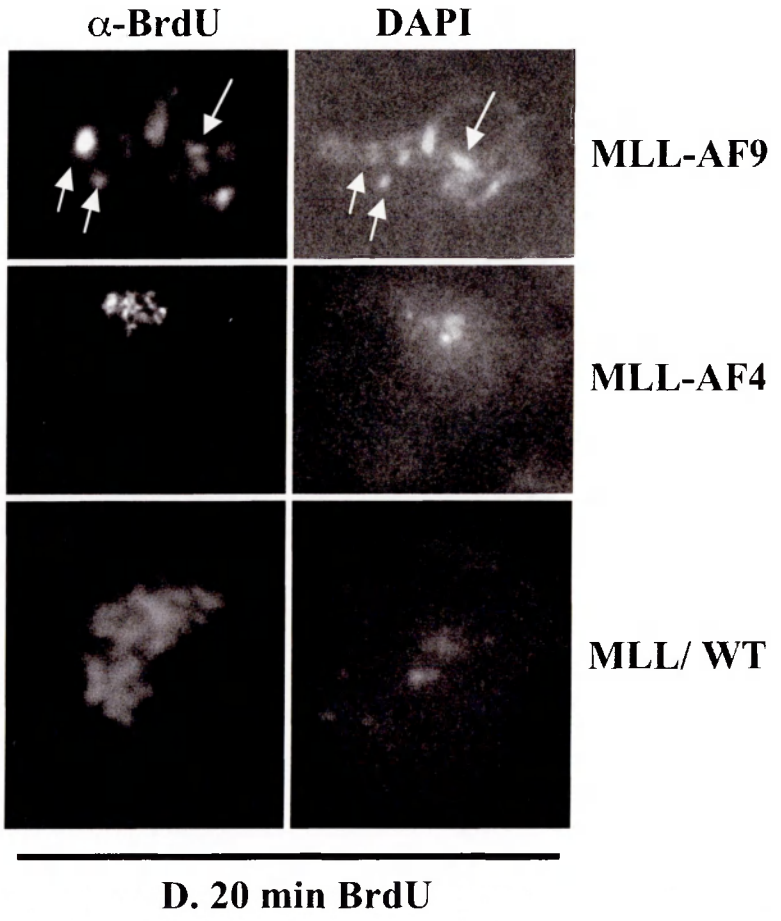


Figure 4.9. *in vivo* BrdU labelling indicates that the presence of MLL-AF9 induces faster mitotic entry. Third instar larvae were fed a mixture of *Drosophila* instant food and BrdU (50 mg/ml) for the indicated times at 29 °C. A 8 hours (A) and 2-4 hours (B) BrdU incubations are sufficient to incorporate BrdU completely into mitotic figures from MLL transgenic lines and wild-type (WT). However, at BrdU exposure of 1 hour (C), mitotic figures from MLL-AF4 and wild-type larvae were not observed to incorporate BrdU, whereas MLL-AF9 and MLL displayed BrdU labelled mitotic figures. As shown, MLL-AF4 exhibited only interphase nuclei labelled with BrdU speckles, possibly corresponding to the nucleolus (arrow-heads). Similar stained nuclei were observed in wild-type brain squash preparations. As shown in this case, mitotic figures in wild-type preparations were clearly not labelled. Interestingly, mitotic figures labelled at heterochromatic regions, such as the centromeres (arrows), were frequently observed in MLL squashed preparations, whereas a completely labelled anaphase was detected in MLL-AF9. (D) 20 minutes of BrdU pulse is sufficient to observe interphase nuclear staining in MLL-AF4, MLL and wild-type, as shown. Only MLL-AF9 exhibited partially labelled mitotic chromosomes (arrows point to incorporated BrdU at the centromeres). These results indicate that MLL-AF9 induces faster mitotic entry.

The *in vivo* BrdU incorporation data indicate that MLL-AF9 is able to initiate DNA replication at a quicker rate than the other transgenic lines and wild-type since a 20 minute pulse of BrdU exposure was sufficient in timing to allow incorporation of BrdU partially into mitotic figures. At 1 hour BrdU exposure, euchromatin of MLL-AF9 mitotic figures had been labelled. This distinction is pertinent because this implies that BrdU was incorporated at the beginning of S phase, and that 1 hour allowed progression of BrdU replicated DNA to reach metaphase. On the other hand, MLL showed heterochromatin labelled figures at this time point, reflecting that BrdU incorporation commenced at the end of the S phase. The fact that no metaphase spread displayed BrdU labelled euchromatin suggests that 1 hour is not sufficient to allow BrdU incorporated DNA at early S phase to reach metaphase. The *in vitro* BrdU incubation of whole brains already hinted that the presence of MLL-AF9 contributed to mitotic entry, since a larger number of replicating cells were observed (Fig 4.7). The finding with *in vivo* BrdU labelling corroborates the *in vitro* data.

Furthermore, timing of DNA replication is not altered relative to chromosomal location in MLL transgenic lines as documented for *orc2* and *orc5* mutants (Loupart el al., 2000). Early replicating euchromatin was observed to be completely labelled with BrdU prior to late replicating heterochromatin at the centromeres. This fact suggests that MLL-AF9 affects aspects of cell cycle progression, other than timing of DNA replication. Possibly, MLL-AF9 may shorten the G2 phase, and thus consequently, allow a larger number of observable BrdU labelled mitotic figures.

4.3 Discussion

4.3.1 *trx* is required to allow cells to replicate in late *Drosophila* development.

To date, it is not known precisely how PcG and trxG members regulate their loci at the mechanistic level. It has been postulated that the formation of appropriate higher order chromatin environment represents an important level of regulatory mechanism by trxG and PcG members (Paro, 1990; Pirrotta, 1998; Francis and Kingston, 2001). The range of *trx* mutants exhibiting various phenotypes (Breen et al., 1999) indicates that possibly PcG and trxG members have a wider repertoir of functions than only the maintenance of proper higher order chromatin at early development. The finding that PcG/ trxG bind to numerous target sites, argues for this speculation (Zink and Paro, 1989; Franke et al., 1992; DeCamillis et al., 1992; Rastelli et al., 1993; Chinwalla et al., 1995; Tripoulas et al., 1996; Francis and Kingston, 2001).

Late lethal *trx* mutants exhibit similar phenotypes as mutants affecting essential cell cycle functions isolated by Gatti and Baker (Gatti and Baker, 1989). The molecular characterisation of several cell cycle mutants, as described above, revealed that DNA replication has been compromised, and that DNA replication mediators are essential for cell cycle progression (Pflumm and Botchan, 2001; Loupart et al., 2000; Krause et al., 2001). The analysis of late lethal *trx* mutants has connected late TRX mediated chromatin regulation with the ability of cells to replicate. Possibly, *trx* impinges directly or indirectly on the DNA replication machinery. Although classically *trx* is thought to keep chromatin structure open, the paradox, that in late *trx* lethals chromatin appears to be incapable of condensing, can be accounted by proposing that *trx* has two distinct functions: (1) modulation of labile chromatin for transcriptional regulation at embryogenesis, and (2) regulation of higher order, structured chromatin present at later stages in development. The first function involves making target genes more accessible for transcription, while the second function is probably associated with making chromatin favourable to replication and thus non-expressible. The possibility that *trx* is involved in both opening and closing of chromatin is not that remote, as several studies have shown that trxG members regulate not only active genes but also repressed loci (Rastelli et al.,

1993; Collins et al., 1999; Kal et al., 2000; LaJeunesse and Shearn, 1996; Gildea et al., 2000; Brock and van Lohuizen, 2001; see Section 1.3.4). The lack of DNA replication in late larval to pupal *trx* lethals implies that probably TRX regulation of structured, higher order chromatin prior to the on-set of S phase is necessary for the completion of *Drosophila* development. The lack of properly formed chromatin structure may have the consequence of preventing correct recognition of origins by the replication machinery. The inability to initiate replication results in accumulation of interphase and "prophase-like" nuclei observed in these *trx* mutant alleles, and suggest that cell cycle progression to metaphase has been impaired, possibly by the activation of cell cycle checkpoints. It has been well established that chromatin environment plays a large role in determining correct transcriptional activity. The results presented here expand the repertoire to include chromatin structure imperative for DNA replication.

4.3.2 MLL-AF9, and not MLL-AF4, appears to interfere with cell cycle progression.

An intriguing outcome from Chapter 4 experiments is the link between chromatin aberrations and defects in replicating cells, as shown with the late lethal *trx* mutants and with MLL-AF9. Importantly, the similar phenotypes exhibited among replicative defective mutants, late lethal *trx* mutants, and MLL-AF9 strongly suggest that TRX and MLL-AF9 compromises DNA replication to account for their effects on cell cycle progression. Two models have been formulated to account for the chromatin defects observed in replication mutants lacking proper DNA replication. Both models propose that DNA replication plays an important role in generating proper chromatin configurations.

The first model considers the impact a lower rate of DNA replication has on chromatin structure (Pflumm and Botchan, 2001). It is postulated that during DNA replication, loops are formed while DNA goes through the replication machinery, forming the first degree of condensation. The notion that the DNA replication machinery remains attached, and consequently would create a defined length of chromosomal contraction, was proposed to avoid entangled DNA during replication (Sundin and Varshavsky, 1980; Hearst et al., 1998). During mitosis, these loops become more tightly coiled creating highly compact chromosomal

structures. In replication defective mutants, the number of functional replication centers is rare. As a result, the model predicts that fewer and larger loops are formed from lack of adequate number in functional replication centers. During mitosis, the coiling of these larger loops create shorter, decondensed, and disorganised mitotic figures.

The second model incorporates replication timing as a factor in generating chromosomal abnormalities (Loupart et al., 2000). In this model, delay of euchromatin replication, as demonstrated from BrdU *in vivo* labelling in replication mutants is linked to the generation of decondensed chromosomal areas. The number of "landing pads" created by replication factors (such as ORCs) is crucial for the proper formation of pre-initiation complex necessary for origin firing. Mutants where amount of replication factors have diminished, influence the number of functional "landing pads" formed, with particular effect at the first DNA origins fired during replication. Euchromatin has been observed to replicate prior to heterochromatin, although exceptions do exist, and the mechanisms behind sequential origin firing are not understood. The model predicts that the inability to form complete "landing pads" at the right time results in regions where DNA is not properly condensed. In areas where DNA replication has failed completely, chromosome breaks would occur, generating "fragmented" metaphase spreads.

How can the data accumulated for MLL-AF9 fit within these two models? The fact that an increase in replicating cells occurs would predict that longer, compact chromosomes should be observed during metaphase according to the first model. MLL-AF9 appears to cause an elongation of chromosomal arms, but definitely, does not compact chromatin, as shown in Fig. 4.3. The second model suggests that hyper-replication would still generate chromosomal decondensed areas since timing of replication, whether early or late, has been compromised. The observation of correct DNA replication timing in MLL-AF9 expressing cells argues against this rationale. Both models do not fully account for the late lethal *trx* mutant's or MLL-AF9's chromatin phenotype. The models rely on misregulation of DNA replication as the main factor generating the chromatin phenotype. It is more likely that in the cases of both late lethal *trx* mutants and MLL-AF9 transgenic fly lines, compromised chromatin architecture influences the ability of DNA replication machinery to faithfully replicate the genome (see Chapter 6). Alternatively, TRX and MLL-AF9 may affect other

aspects, other than the DNA replication machinery, to directly account for their effects on cell cycle progression.

Although MLL-AF4 displayed pairing defects (an indication that the chromatin structure "glue" has been altered) in polytenes as MLL-AF9, the effect of MLL-AF4 on chromatin integrity in diploid cells was not detectable. Unlike MLL-AF9, the molecular similarities between MLL-AF4 and replication defective mutants were non-existent, indicating that MLL-AF4 induces fly lethality by interacting with another pathway(s) other than DNA replication. In addition, the observation that metaphase spreads from colchicine-treated brain squashes resembled wild-type both in morphology and in chromosome number, could not confirm results from experiments detecting polytene-like structures in MLL-AF4 expressing cells (Chapter 3). The lack of endoreduplicated cells in MLL and in MLL-AF4 expressing cells implies that the polytene-like structure documented in Chapter 3 may in fact be rare cases of uncontrolled proliferation that have no bearing on the viability of the fly. In this respect, it has been reported that *efe*^{89}, an interactive partner with MBT (Malignant Brain Tumour) mutant, display up to 20% of polytene-like structures in the brain (Reide, 1996b). Only 1-2 polytene-like structures were found in MLL and in MLL-AF4 transgenic flies.

Combining the subtle chromatin effect associated with MLL-AF4 with the clear chromatin abnormalities induced by MLL-AF9, it is tempting to postulate that maintenance of chromatin structure may be a general functional property of MLL fusion proteins. The finding that both MLL-AF4 and MLL-AF9 transgenic fly lines are associated with a higher number of BrdU positive cells in a short BrdU exposure time indicates that cell cycle proliferation has been compromised. Possibly, this effect is a consequence of altered chromatin structure induced by MLL fusion proteins. The putative, global modulation of chromatin by MLL fusion proteins varies to the extent this property is manifested as illustrated in this chapter for MLL-AF4 and MLL-AF9. In this respect, additional functions specifically contributed by the C-terminal partner would have a crucial role in deregulating additional defensive mechanisms necessary for leukemogenesis. Therefore, a first step in leukemogenesis may be the predisposition of cells by creating a more labile-chromatin structure, which would then allow additional functions specifically associated with individual MLL fusion proteins to deregulate cell cycle control. In the case of MLL-AF9, the

acceleration of cells replicating may facilitate the accumulation of secondary mutations by over-saturating the safeguard mechanisms maintaining correct replication, and thus, bypassing cell cycle checkpoints. The aftermath would be the generation of pools of cells that intrinsically harbour mutations, for which some allow the onset of leukaemia. On the other hand, the characterisation of the *Drosophila* AF4 homolog, *lilli*, points MLL-AF4 acting on pathways regulated by Ras/MAPK and by PI3/PKB and affecting cell size growth (Wittwer et al., 2001; Tang et al., 2001). The extensive similarities between *Af4* null knock-out in mice and the *Drosophila lilliputian* mutant suggest that conservation exists in the regulation of cellular growth (Isnard et al., 2000; Wittwer et al., 2001).

CHAPTER 5

MLL and MLL fusion proteins exhibit distinguishing binding patterns on polytenes.

5.1 Introduction

The PcG and trxG members are regulators of gene expression, acting on higher order chromatin fibers. It is not known how members of both families recognise their target elements. It is speculated that few PcG/ trxG members are able to contact DNA directly, and that specificity is endowed through protein-protein interaction (Paro 1990; Pirrotta, 1998; Francis and Kingston, 2001). Alternatively, PcG/ trxG may recognise higher order chromatin configurations that are dependent on the DNA sequence. The formation of particular loops by the refolding of DNA onto itself may actually be the central recognition module for PcG/ trxG members (Francis and Kingston, 2001).

Defining the DNA sequences, which are bound by PcG and trxG members, along the genome may unveil consensus elements recognised by PcG/ trxG. For this, *Drosophila* offers the advantage of staining polytenes. Polytenes are special structures found in the salivary glands. These "giant chromosomes", as they are sometimes referred to, are consequences of continuous replication of the genome in the absence of mitosis. The net result is the formation of tubular structures composed of 1000-2000 genomes juxtaposed to one another to form bands along the arms of the chromosomes. The bands correlate to genes. Their locations along the arms of the chromosomes have been previously determined by *in-situ* hybridisation against sequences spanning the target genes. Importantly, polytene staining allows mapping of protein distribution and identification of target genes. It has been shown by using antibodies specific to several PcG and trxG proteins on polytenes that these regulatory proteins specifically bind to a limited number of elements (Zink and Paro, 1989; Franke et al., 1992; DeCamillis et al., 1992; Rastelli et al., 1993; Chinwalla et al., 1995; Tripoulas et al., 1996). These binding patterns of several PcG and trxG members have also demonstrated that PcG and trxG members share overlapping sites, suggesting that an enormous pool of different complexes exist to properly regulate gene expression.

An interest in the mammalian field is to define the genomic distribution of MLL and of MLL leukaemic proteins, since to date, evidence is lacking in this respect. Polytene staining against MLL would provide the first clues as to which target genes may be regulated by MLL. The identification of these target regulatory elements on polytenes would thus provide insight

into the undertanding of MLL's vital function(s) in cell regulation. The strong conservation of developmental pathways between *Drosophila* and mammals indicates that the targets identified in the fly will likely have relevance in mammals (see Section 1.2). Defining how the binding pattern of MLL compares with the distribution of TRX on polytenes will also reveal whether MLL can incorporate or even interchange with TRX in physiologically relevant complexes.

Most importantly, polytene stainings against MLL and MLL fusion proteins would indicate if the new AF9 and AF4 C-terminal sequences perturb MLL protein targeting. Chapter 4 has provided evidence demonstrating that MLL-AF9 appears to act through a late TRX function in derailing cell proliferation. Data thus far points to MLL-AF9 as possessing unique properties halting *Drosophila* development. Thus, it is expected that MLL-AF9 controls different target genes as compared to MLL. Additionally, the strong lethality observed by MLL-AF4 points to a different protein distribution from MLL. Because MLL-AF4 does not interfere with the same pathway as MLL-AF9 to inhibit fly viability, it is conceivable that both MLL fusion proteins also display distinguishing binding patterns.

Chapter 5 presents evidence demonstrating that MLL cannot simply replace TRX, and that MLL-AF9 and MLL-AF4 distribute differently on polytenes. The chapter begins by describing the generation of an affinity-purified TRX antibody. This antibody in conjunction with an affinity-purified antibody against the N-terminal region of MLL have been used on polytenes to gain insight into the molecular behaviour of MLL and of MLL fusion proteins in respect to TRX. Furthermore, co-staining of polytenes against Polycomb (the prototype of the PcG family) and N-terminal MLL antibodies suggest that MLL and MLL fusion proteins do not interact, and possibly may not require PcG/ trxG members for their functions. The distribution of MLL and of MLL fusion proteins, differ considerably and have implications on modifications of chromatin. Finally, genetic experiments aimed to ask if MLL can functionally replace TRX indicate that inter-changeability between species is not so straight-forward. These results are discussed on the basis of the molecular mode of action(s) employed by MLL and by MLL fusion proteins, and consider their relevancy to the histone code.

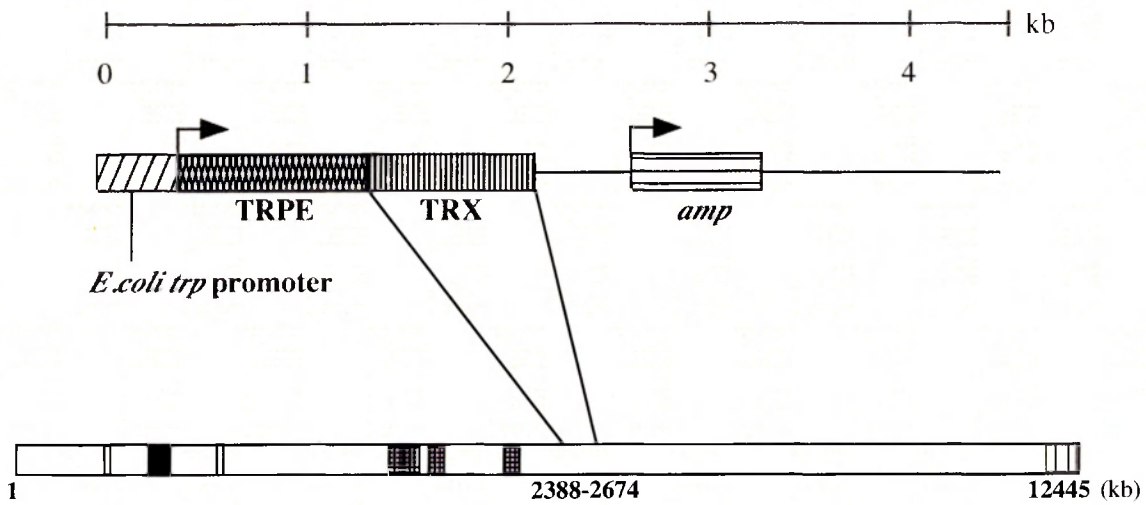
5.2 Results

5.2.1 Generation of affinity pure TRX antibody

The TRX residues spanning 2388-2674 was used to generate affinity pure TRX antibodies. Both short (TRX I) and long (TRX II) isoforms of TRX are recognisable by the antibody. The region was cloned into the pATH III vector (Fig 5.1a), which allows production of large amounts of fusion proteins under the tryptophan inducible system (Koerner et al., 1991). The tryptophan system remains tightly inactive unless tryptophan or its analogue, indoleacrylic acid (IAA), is present. IAA was used to induce the activation of the *E.coli trp* operon promoter, by preventing the binding of the *trp* repressor (Koerner et al., 1991). The fusion protein, TRPE-TRX with the expected size of 68 kDa, was produced in high amounts when 2 mg/ml IAA was added to the culture medium (Fig 5.1B, compare lane 3 vs. lane 4). The insoluble TRPE-TRX fusion protein was purified to homogeneity by gel elution (Fig 5.1B, lane 2). The purified protein was subsequently used for injections into rabbits.

IgG antibodies were purified from 6th boost serum using a Protein A sepharose column. The purified antibody, designated Prot A-TRX, was found to detect 100 ng of the TRPE-TRX fusion protein at a 1:10,000 dilution (Fig 5.2A), and was tested on nuclear extract from overnight lays of *Drosophila* embryos. As shown in Figure 5.2B, TRX of estimated size 300-400 kDa was recognised. Various TRX antibodies have been previously reported to detect degraded forms of TRX, such as the 180 kDa band (Kuzin et al., 1994). These Western blot analyses reveal that under these conditions, the ProtA-TRX is able to efficiently detect TRX with minimal cross-reactivity against unspecific proteins.

A.



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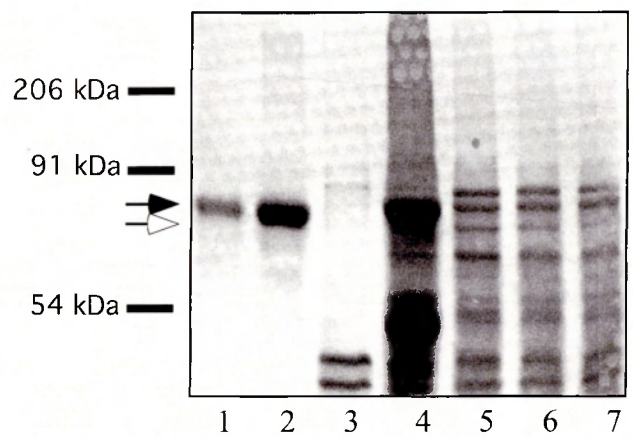
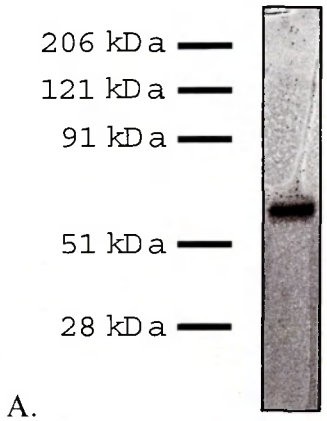
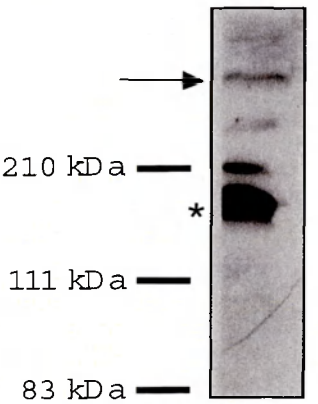


Figure 5.1. Induction of TRPE-TRX fusion proteins for generation of antibodies. (A) Schematic representation of the pATH III vector used to express the TRPE-TRX fusion protein (approximately 68 kDa). As shown, the residues corresponding to 2388-2674, lie at an unconserved region of the TRX protein sequence. (B) Coomassie analysis for induction and estimation of protein production after gel purification. Lanes are designated as the following: 1.BSA (dark arrow), 2. purified TRPE-TRX (open arrow), 3. no induction of TRPE-TRX protein, 4-7. four different TRPE-TRX clones induced with 2 mg/ml IAA. Clone from lane 4 was used further to induce and to purify additional fusion proteins.



A.



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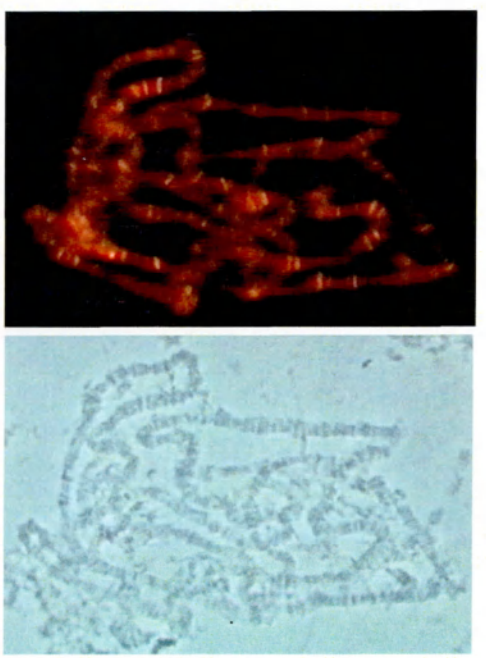

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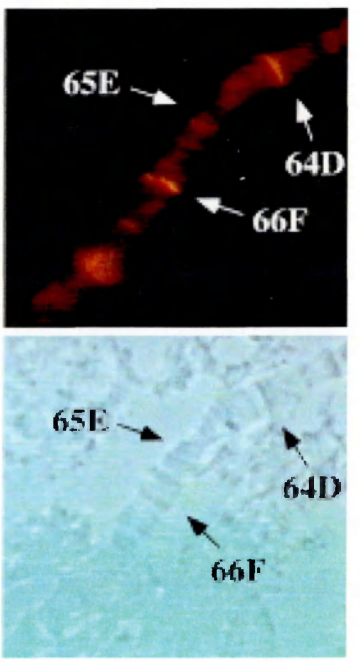


D.

Figure 5.2. Protein A-TRX antibodies detect TRX in denatured and in native conditions. (A) Detection of TRPE-TRX fusion protein (100 ng) at 1:10,000 dilution. (B) Detection of full-length TRX (300-400 kDa) at 1:5,000 (200 µg of nuclear extract was loaded). The lower bands below correspond to TRX degradation products. Similar degradation products (*) have been reported (Kuzin et al., 1994). (C) Staining of wild-type polytenes with Protein A-TRX antibodies at 1:200 dilution (40x magnification). (D) The 64-66 region has been reported to be bound by TRX, as shown (100x magnification) (Chinwalla et al., 1995).

Since the same construct was used to previously generate antibodies (Chinwalla et al., 1995), the availability of the TRX binding pattern provides an ideal control for testing of the newly generated antibodies on polytenes. As Figure 5.2C details, the antibody strongly sees around 80 bands, supporting previous data (Chinwalla et al., 1995). Higher magnification analysis also demonstrates that the ProteinA-TRX antibodies specifically detect TRX, since TRX has been previously documented to bind 64D, 65E, and 66F (Fig 5.2D).

To ascertain if all bands recognised by Protein A-TRX reflect TRX binding, two *trx* mutants (trx^{JY16} and trx^{E3}) were crossed to create trx^{JY16}/trx^{E3} progeny. Both trx^{JY16} and trx^{E3} mutants possess deleted portion of the TRX sequence used to generate the antibodies. Therefore, it is expected that no bands should be detectable in double mutant larvae with a specific TRX antibody. As shown in Fig 5.3, ProteinA-TRX antibodies recognised many unspecific proteins.

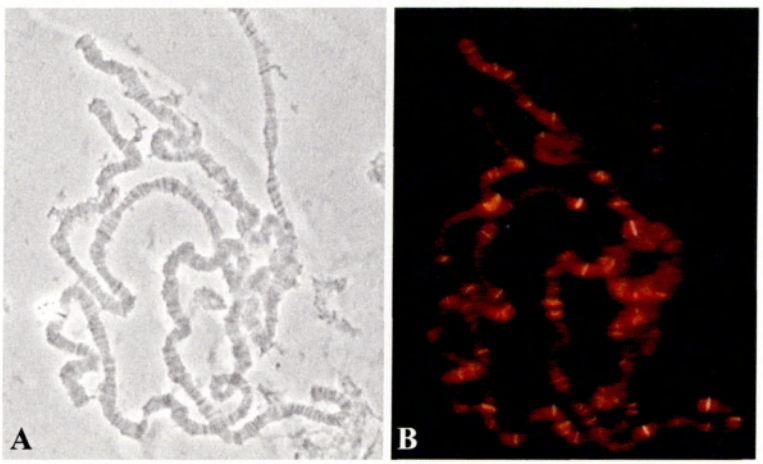
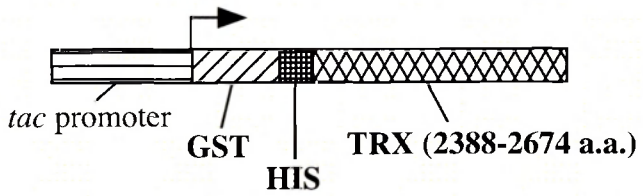


Figure 5.3. ProteinA-TRX antibodies recognise unspecific proteins. Bands in polytenes from trx^{JY16}/trx^{E3} mutant larvae should not be detectable by a specific TRX antibody generated against the 2388-2674 residues of TRX. As shown, Prot A-TRX serum cross-react with numerous unspecific bands. A. Phase contrast. B. Cy3 secondary antibody against rabbit ProtA-TRX antibodies.

To purify antibodies specific against TRX and not against the TRPE portion of the fusion protein, the TRX sequence spanning 2388-2674 residues was cloned into the pGEX-4T3 vector using ET cloning. The pGEX-4T3 allows production of GST-HIS tag N-terminal to the TRX sequence (Fig 5.4A). The Protein A-TRX antibody detected the GST-HIS-TRX fusion protein. The new, insoluble GST-HIS-TRX was purified in 6 M Urea over a Ni^{2+} column (Fig. 5.4B). Purified, soluble GST-HIS-TRX in non-denaturing conditions was bound onto nitrocellulose, renatured, and then used to affinity purify TRX antibodies from ProteinA-TRX serum (see Section 7.2.1). Two batches of antibodies were eluted. The first batch (Aff#1-TRX) consistently gave similar unspecific bands as for ProteinA-TRX antibodies on polytenes from trx^{JY16}/trx^{E3} double mutants (Fig 5.5A). On the other hand, a significant reduction of detectable signals was observed with the second batch of TRX antibodies (Fig 5.5B). This batch, Aff#2-TRX, was used for subsequent antibody stainings, and is referred to as α-TRX.

A.



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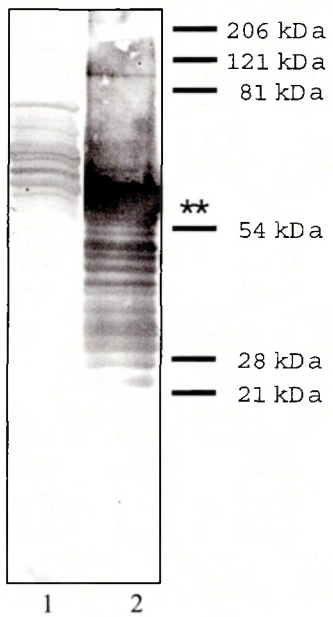

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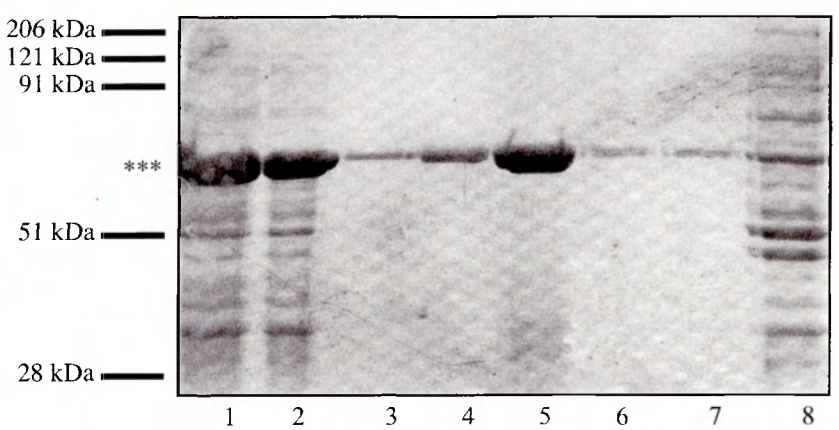


Figure 5.4. Induction and purification of GST-HIS-TRX. (A) Schematic representation of the fusion protein, GST-HIS-TRX, expressed from the pGEX-4T3 vector (Pharmacia). (B) GST-HIS-TRX (**, approximately 59 kDa) is detectable by Protein A-TRX antibodies (1:3,000). Lanes: 1. GST-HIS bacterial lysate; 2. GST-HIS-TRX bacterial lysate. (C) Coomassie stained SDS-PAGE gel illustrating the purification of insoluble GST-HIS-TRX protein. GST-HIS-TRX (***) was purified to homogeneity over a Ni^{2+} column. Lanes: 1. induced GST-HIS-TRX; 2. GST-HIS-TRX bound to Ni^{2+} beads; 3. Elution Fractiond No. 4; 4. Elution Fraction No. 3; 5. Elution Fraction No. 2; 6. Wash Step No. 1; 7. Wash step No. 2; 8. Flow through.

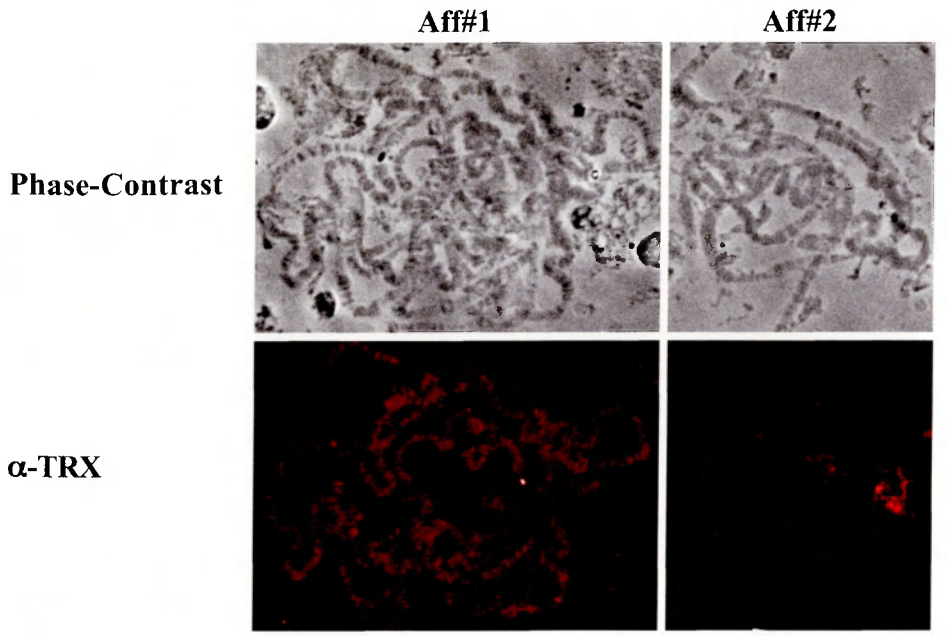


Figure 5.5. Aff#2-TRX antibodies reduce unspecific signals to background levels on polytenes from *trx*^{JY16}/*trx*^{E3} double mutants. Polytene staining with Aff#1-TRX eluted antibodies reveals a persistent detection of unspecific bands on polytenes from *trx*^{JY16}/*trx*^{E3} mutants. The reduction of these signals to background levels was observed with Aff#2-TRX purified antibodies. Aff#2 TRX antibodies (designated α-TRX thus forth) were used for subsequent antibody stainings.

5.2.2 MLL, MLL-AF9 and MLL-AF4 display distinguishing protein binding profiles, and co-localise minimally with TRX or Pc on polytenes.

The MLL and MLL-AF9 sequences are tagged with FLAG epitope at their C-terminus (see Figure 2.3). The FLAG monoclonal antibody (Sigma) and a specific monoclonal antibody against the C-terminal AF4 protein were used in conjunction with α-TRX antibodies to explore if MLL or MLL fusion proteins co-localise with TRX on polytenes. Expression of MLL, of MLL-AF9, and of MLL-AF4 under the control of a heat shock promoter, was induced by heat shocking third instar larvae for 30 minutes and then allowing their recovery at 18 °C for at least 5 hours. As shown in Fig. 5.6, limited co-localisation, around 10%, was detected between MLL and TRX. MLL-AF9 and TRX were mutually exclusive in binding pattern while MLL-AF4 rarely co-localised with TRX.

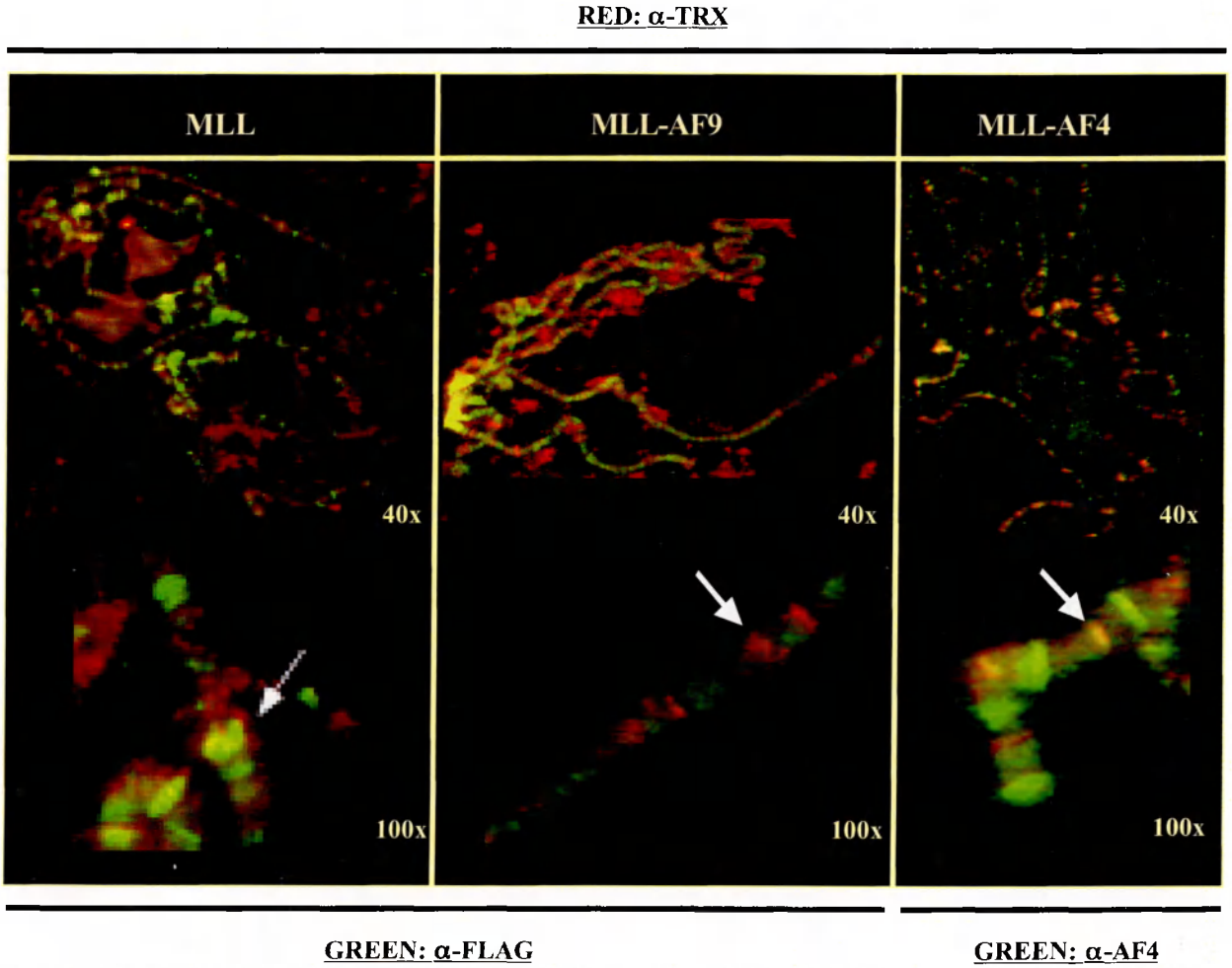
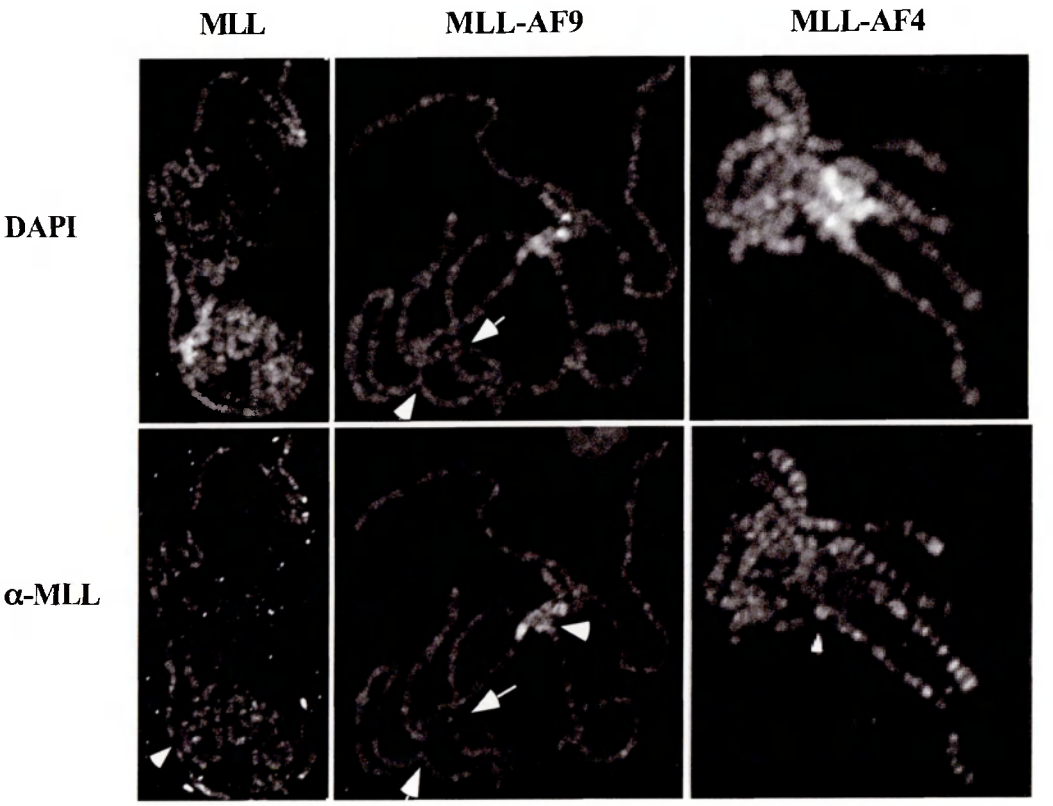


Figure 5.6. MLL and MLL-AF4 distinctly co-localise with TRX at limited sites on polytenes, whereas MLL-AF9 and TRX bind mutually exclusively. Double labelling of polytene chromosomes demonstrates that MLL-AF9 and TRX do not co-localize, while MLL and MLL-AF4 only minimally co-localise with TRX. TRX is detected with polyclonal antibodies (red), whereas MLL and MLL-AF9 are detected with monoclonal antibodies to FLAG (green), and MLL-AF4 with monoclonal antibodies to AF4 (green). Polytenes on the top were captured at 40x magnification, whereas lower images are at 100x magnification. MLL was found to bind strongly at limited sites with TRX (left top). Similarly, MLL-AF4 co-localised with TRX at much fewer bands than MLL and with a much weaker intensity, possibly reflecting that the bindings by TRX and MLL-AF4 at the region is close but not superimposed (right, top). However, MLL-AF9 (green) and TRX (red) bound to polytenes exclusively from each other (middle, top). As clearly shown in the lower images (arrows), MLL was found to co-localise strongly with TRX at cytological location 2C (left, bottom). At the same cytological position 2C, MLL-AF9 was not bound, indicating that the fusion protein binds to sites different from MLL (middle, bottom). MLL-AF4, however, appeared to weakly co-localise with TRX at 2C. These binding patterns indicate that MLL fusion proteins are targeted to different sites on polytenes, and suggest that MLL cannot fully incorporate into all TRX complexes.

Since the generation of protein expression by heat shock may reflect unspecific binding by excessive protein levels, polytene stainings were performed from larvae expressing MLL, MLL-AF9, and MLL-AF4 with the daughterless Gal4 driver at 29 °C, to confirm that the transgenic protein binding patterns are relevant. Importantly, under this condition, MLL-AF4 and MLL-AF9, but not MLL, are able to induce the lethal phenotype. A specific N-terminal MLL (α-MLL) antibody was used to detect the transgenic proteins. Remarkably, MLL, MLL-AF9 and MLL-AF4 displayed distinct, unique binding patterns (Fig. 5.7). A general distribution of MLL throughout the chromosomes of polytenes was observed. On the other hand, MLL-AF9 was found at limited euchromatic sites, and intriguily, at the heterochromatic-rich chromocenter. The binding profile of MLL-AF4 drastically differed from MLL and from MLL-AF9 since strong, defined bands were observed regularly throughout the polytene arms. The binding patterns of MLL and of MLL fusion proteins in a scenario where protein levels should be constant (as in this case when the daughterless Gal4 driver is used), were similar to the above staining patterns for MLL, MLL-AF4, and MLL-AF9 induced by heat-shock (Fig 5.6), and thereby justify the results presented at Fig. 5.6. Furthermore, α-TRX staining of polytenes from transgenic lines induced with daughterless-Gal4 driver, show that MLL and MLL fusion proteins do not alter the TRX binding pattern (Fig 5.7).

A.



B.

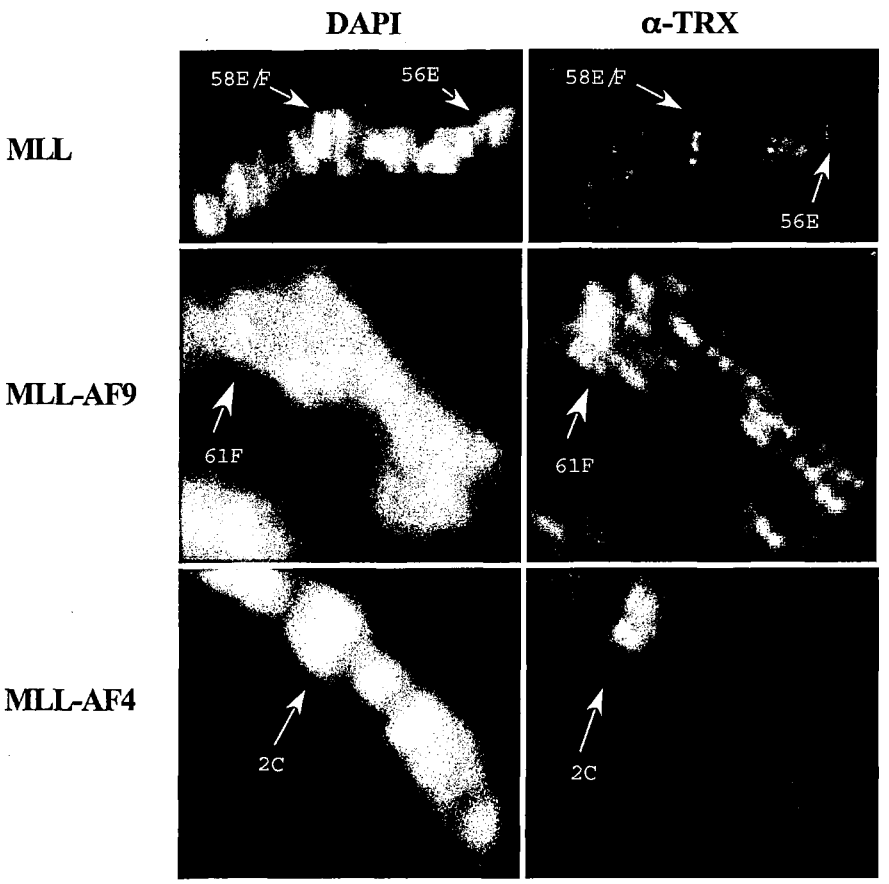


Figure 5.7. Stainings of polytenes, from salivary glands expressing MLL, MLL-AF9, or MLL-AF4 with the daughterless Gal4 driver, uncover distinguishing binding patterns for each transgenic protein and show that TRX binds to its target sites regardless of the presence of the transgenic proteins. (A) Staining with polyclonal antibodies against MLL (α-MLL) demonstrated that the binding profile of MLL-AF9 and MLL-AF4 differ considerably from MLL. As shown, MLL was found bound throughout the chromosomes, with limited defined banding. Interestingly, MLL-AF9, but not MLL nor MLL-AF4, was largely concentrated at the heterochromatin-rich chromocenter (middle, arrow head). On the other hand, MLL-AF4 binding pattern confined to many bands along the chromosome arms, and not to the chromocenter. (right, arrow head). The arrows in MLL-AF9 point to polytene paring defects (see Fig 3.4). Images are illustrated at 40x magnification. (B). Staining with α-TRX antibody on polytenes from MLL, MLL-AF9, and MLL-AF4 confirmed that TRX binding is not affected by the presence of the transgenic proteins. Indicated bands in (B) have been reported to be bound by TRX (images at 100x maginification and Kuzin et al., 1994; Chinwalla et al., 1995).

Although the polytene stainings above show that MLL, MLL-AF9, and MLL-AF4 do not co-localise with TRX significantly, MLL may still be able to interact with other members of PcG and trxG to form functional complexes, while MLL fusion proteins may aberrantly alter PcG/trxG binding patterns. To explore this idea, the Polycomb (Pc) protein was selected since not only is it the prototype of the PcG family, but it also shares overlapping binding sites with TRX (Kuzin et al., 1994; Chinwalla et al., 1995). Double labelling experiments with mouse Pc antibodies and the N-terminal MLL polyclonal antibodies revealed that Pc does not overlap with any of the transgenic lines (Fig 5.8). These results support experiments showing that MLL and TRX rarely co-localise, and imply that MLL fusion proteins may not require the association other PcG/ trxG members to induce lethality.

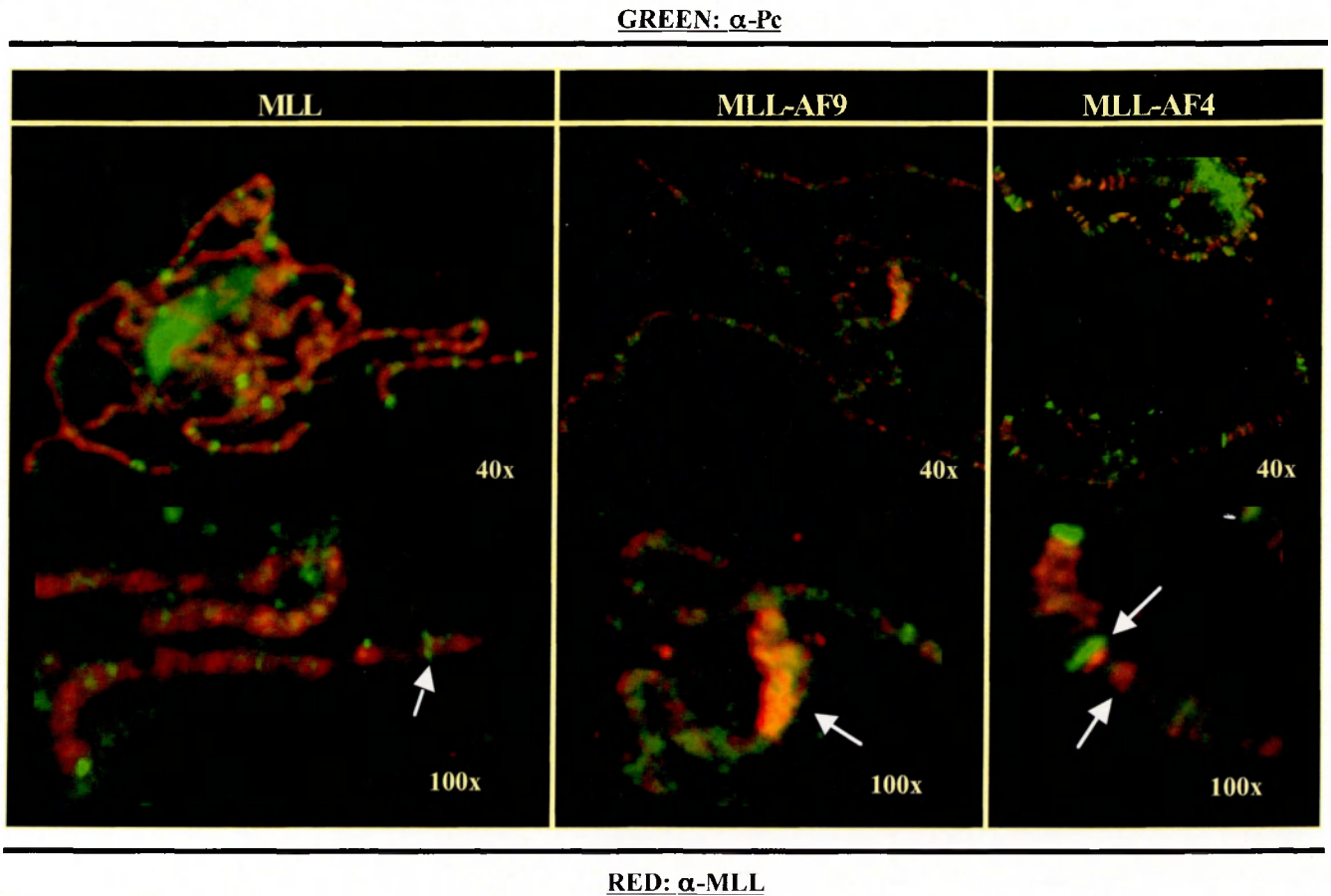


Figure 5.8. Pc and transgenic proteins do not co-localise on polytenes. MLL, MLL-AF9, and MLL-AF4 were detected using the N-terminal MLL antibody. Double labelling stainings for Pc (green) and MLL, MLL-AF9, or MLL-AF4 (red) illustrate that in all cases, the proteins do not co-localise (arrows).

5.2.3 MLL cannot simply rescue *trx* mutants

The finding that TRX and MLL only partially co-localise was validated by genetic experiments aimed at defining the extent MLL can functionally replace TRX. The trx^{JY16} and trx^{B11} mutants (as described in Chapter 4) were used. The *trx* mutants carry additional markers that allow genetic manipulation to be followed. In this manner, fly stocks can be generated to include both the *trx* mutant and the appropriate MLL transgenic construct. A crossing scheme allowed the expression of MLL in a *trx* heterozygous or homozygous mutant background, and investigated if MLL expressing progenies are able to affect viability of flies lacking half the dosage of wild-type *trx* (as in the case of the heterozygous state), or are able to survive past larvae into pupae or adults (as in the case of the homozygous state). Since a sufficient amount of TRX proteins are present to allow embryonic survival, use of a Gal4 driver expressing high levels of MLL at later stages of development would be most reasonable. The blood MZ1580 and Act5C-Gal4 were chosen as Gal4 sources, since they have been shown to produce high levels of Gal4 at larval and pupal stages (unpublished results; Ito et al., 1997). Corroborating results on polytenes, no rescue of *trx* double mutants was observed by MLL under both conditions (Fig 5.9). Moreover, presence of high levels of MLL did not affect flies lacking half the dosage of wild-type TRX (Fig 5.9). Both the polytene staining and genetic experiments indicate that lack of rescue of *trx* mutants by MLL appears to be due to MLL's inability to incorporate into a sufficient number of functional TRX complexes, or that MLL behaves as an inert protein in *Drosophila*. Therefore, at this level, MLL and TRX are not interchangeable entities.

Identical crosses were performed for MLL-AF4 and MLL-AF9 to assess the role TRX may have on the lethal phenotype induced by the fusion proteins. As shown, reduction or lack of TRX did not affect MLL fusion lethality (Fig. 5.9). These results indicate that a gain-of-function TRX activity appears to not be the cause for the lethal phenotype induced by the MLL fusion proteins.

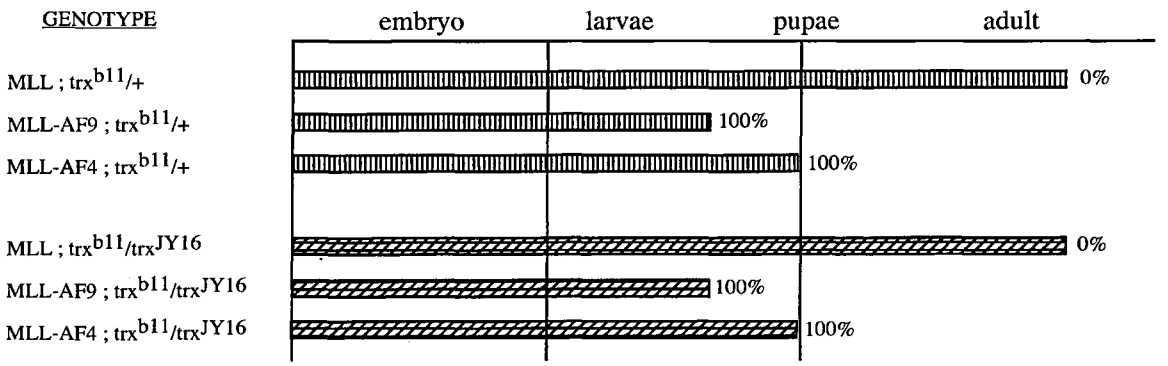


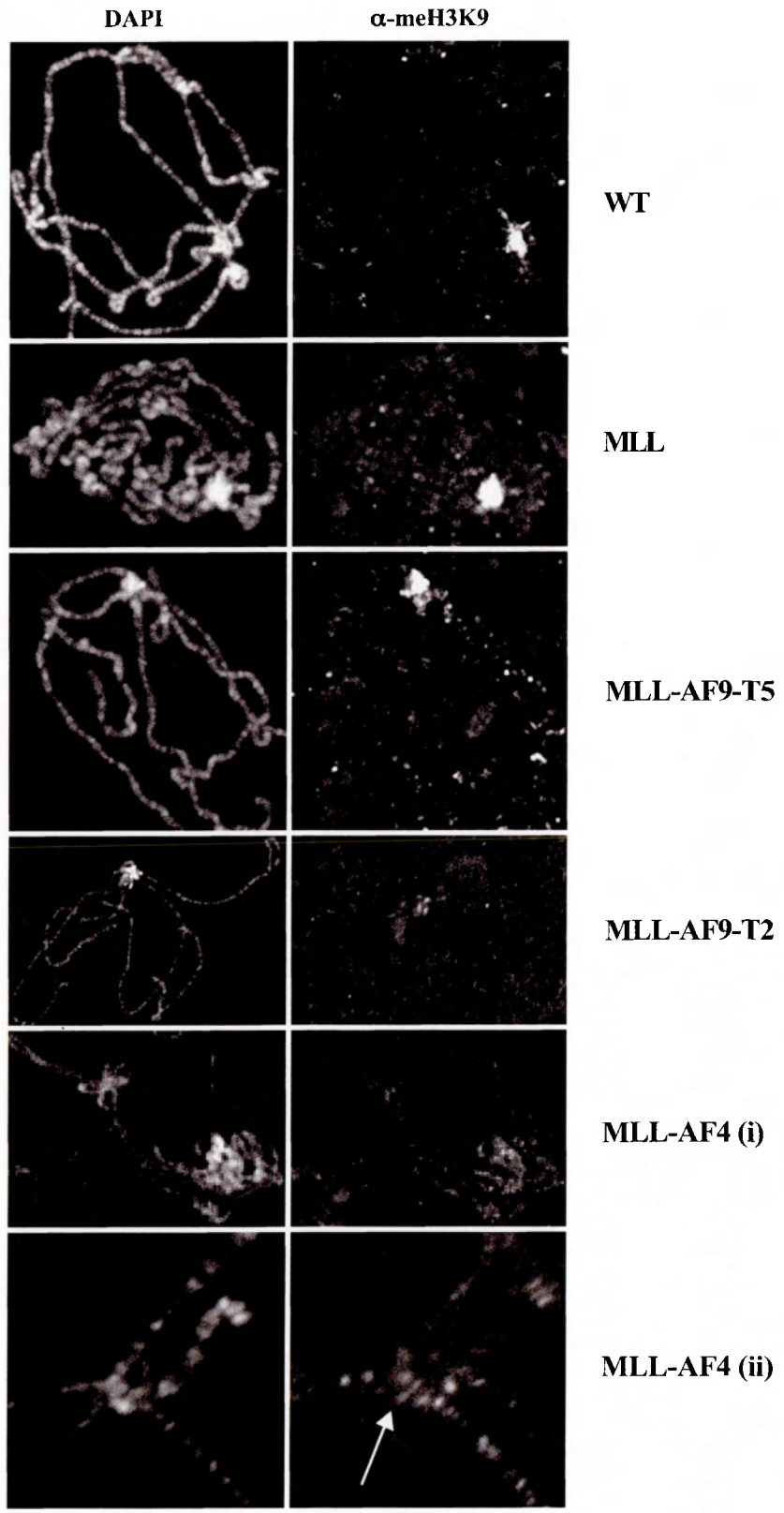
Figure 5.9. Genetic experiments reveal that MLL fusion protein induced lethality is not affected by TRX dosage, and that MLL cannot functionally exchange with TRX. To define more precisely the effect TRX has on the lethal effect induced by the MLL fusion proteins, *trx* alleles (*trx*^{b11} and *trx*^{E3}) were crossed in. The MLL and MLL fusion proteins were expressed with the blood MZ1580 Gal4 driver and with the late expressing Gal4 driver line under the control of a strong Actin5c promoter. The use of both Gal4 drivers did not alter the outcome of the rescue experiments depicted above. As shown, reducing half the amount of functional TRX or omitting TRX does not affect the lethal phenotype induced by the MLL fusion proteins. In addition, it was observed that MLL could not rescue the lethal phenotype associated with *trx* double or single mutants, suggesting that MLL at this experimental set-up cannot recapitulate TRX function(s). Bars represent the stage to which progenies with the indicated genotype (left) developed, whereas percentage reflects the portion of progenies lethal at the defined stage.

5.2.4 MLL-AF9 and MLL-AF4 disrupt the distribution of methylated histone H3 at Lysine 9

The finding that MLL-AF9 concentrates at heterochomatic chromocenter suggested an effect on methylated histone H3 at Lysine 9 (me-H3K9), since this type of modification was shown to mark heterochromatin. Polytene stainings, with specific me-H3K9 antibodies, demonstrated that in polytenes from wild-type larvae, me-H3K9 is predominantly found at the chromocenter and at distinct and limited sites in euchromatin (Fig. 5.10). When polytenes from salivary glands expressing MLL by the daughterless Gal4 driver were probed with the me-H3K9 antibodies, the wild-type pattern was observed (Fig. 5.10). MLL-AF9 polytenes,

generated from conditions inducing complete lethality, illustrated that the chromocenter remained marked by me-H3K9, and significantly, the me-H3K9 bands at euchromatic sites were lost (Fig. 5.10). Strikingly, the reciprocal was found for MLL-AF4 polytenes under lethal conditions (Fig. 5.10). The chromocenter lost me-H3K9 staining, although euchromatic me-H3K9 signals remained. The effect on me-H3K9 pattern by MLL fusion proteins is partially reminiscent of the binding patterns on polytenes for MLL-AF9 and MLL-AF4 (compare Fig. 5.7 and Fig. 5.10). These observations reinforce that MLL-AF4 and MLL-AF9 regulate higher order chromatin structures and suggest that both proteins employ similar mechanisms to deregulate chromatin integrity.

A.



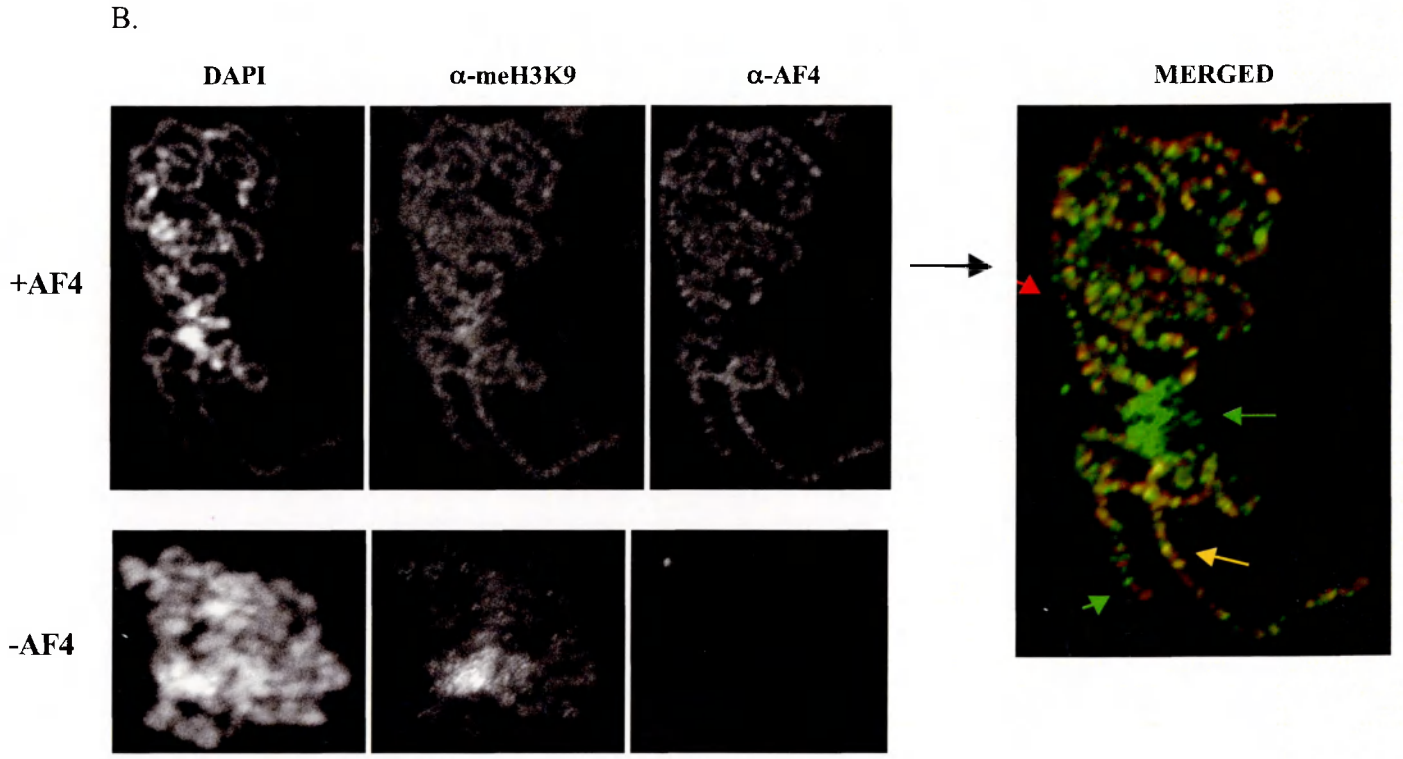


Figure 5.10. The methylation pattern of histone 3 at lysine 9 is affected by the presence of MLL fusion proteins, but not by full-length MLL. The pattern of methylation of histone 3 at lysine 9 (me-H3K9) on polytenes of wild-type larvae is confined to the chromocenter and to limited sites at euchromatin (A, WT). The wild-type me-H3K9 pattern was found to be retained when MLL is expressed in the fly (A, MLL). However, expression of MLL-AF9 caused loss of methylated sites at euchromatin (A, MLL-AF9-T2). This effect was found to be dependent on protein levels since a transgenic line, producing a lower amount of MLL-AF9 and associated with a less severe lethality, retained the wild-type me-H3K9 pattern (A, MLL-AF9-T5). Contrary to MLL-AF9, MLL-AF4 reduces me-H3K9 at the chromocenter and enhances me-H3K9 at euchromatic sites (A, MLL-AF4 (i)). A closer look at the chromocenter confirms that the effect is present (A, arrow MLL-AF4 (ii)). Interestingly, double labelling of polytenes with both AF4 and me-H3K9 antibodies demonstrates that me-H3K9 and MLL-AF4 at the euchromatic arms do not co-localise perfectly (B, +AF4). As indicated in the MERGED image, bands modified by me-H3K9 did not have MLL-AF4 bound (green arrow), although for several bands co-localisation was observed (yellow arrows). Likewise, MLL-AF4 was found bound to polytene bands without an apparent modified H3 (red arrow). The effect on me-H3K9 by MLL-AF4 is specific since polytenes lacking MLL-AF4 exhibit normal me-H3K9 distribution (B, -AF4). (magnification: 40x)

5.3 Discussion

Drosophila offers a unique advantage to look at the distribution of nuclear proteins with relative ease. The polytenisation of cells in the salivary glands allows detection of protein binding profiles since the genome has been replicated thousands of times without mitosis. Since bands on polytenes span over 100kb, co-localisation by two proteins on polytenes does not necessarily reflect that they act in a complex. However, the co-localised binding at many chromosomal sites provides a strong indication that the two proteins may interact with one another. This chapter has presented evidence demonstrating that MLL and MLL fusion proteins display distinct binding profiles on polytenes. Importantly, these different binding profiles indicate that MLL-AF9 and MLL-AF4 employ different target sites to disrupt developmental progression. The finding that MLL-AF9 and MLL-AF4 affect methylation of histone H3 at lysine 9, supports previous data indicating that MLL fusion proteins alter higher order chromatin structure (Chapter 3 and Chapter 4), and extends the idea by offering modulation of histone modifications as a common venue through which MLL-AF4 and MLL-AF9 may accomplish their tasks. In addition, the finding that MLL fusion proteins, as well as MLL, cannot integrate into Pc or TRX complexes suggest that MLL fusion proteins may not require PcG/ trxG association for their function, and that MLL cannot incorporate into PcG/ trxG complexes. The later aspect is supported by genetics showing that at the specific experimental condition, MLL cannot functionally replace TRX.

An important question that remains unanswered in the mammalian field is whether MLL fusion proteins modulate genes regulated by MLL. Using an affinity-purified antibody against the N-terminal portion of MLL, MLL, MLL-AF4, and MLL-AF9 were found to display unique protein binding patterns. This finding correlates well with previous data (Chapter 2-4) demonstrating that MLL fusion proteins adversely affect fly viability while MLL does not, and that MLL-AF9 and MLL-AF4 do not display identical phenotypes (such as the chromatin aberrations reported at Chapter 4). An implication from the distinct binding patterns between MLL-AF4 and MLL-AF9 is that likely, MLL-AF4 and MLL-AF9 target additional pathways other than those regulated by MLL, to eventually deregulate the cell cycle. In this respect, MLL fusion proteins may modulate the activities of new target genes defined by the AF4 and the AF9 sequences.

Interestingly, the localisation of MLL-AF9, but importantly not MLL-AF4 nor MLL, at heterochromatin-rich chromocenter, suggested that methylation of H3K9 may be utilised as a means to alter higher order chromatin, since it has been shown that this histone modification is important for cell cycle progression (see Section 1.3.6). Indeed, the binding profile of methylated H3K9 in MLL-AF9 and in MLL-AF4 reflects the protein distribution of MLL-AF9 and of MLL-AF4, although complete co-localisation of MLL fusion proteins and methylated H3K9 is not observed. This effect on methylated H3K9 appears to be relevant since lack of MLL-AF4 or lower doses of MLL-AF9 do not alter the binding pattern of methylated H3K9. Therefore, the analyses of these polytene stainings provide the first hints that the histone code is a vital pathway through which chromatin regulators may derail cell cycle integrity.

The co-localisation experiments between MLL or MLL fusion proteins and TRX or Pc, unveiled that MLL and MLL fusion proteins did not significantly co-localise with TRX and Pc. MLL fusion proteins may not be able to interact with PC and with TRX as a result of their C-terminal sequence or of the lack of conserved domains at their MLL N-terminus. The data presented in this chapter accounts for both explanations since the binding patterns of MLL-AF9 and of MLL-AF4 differ so extensively with each other. The TRX binding profile appears to be not altered in MLL-AF9 and in MLL-AF4 transgenic flies, again supporting that the new AF9 and AF4 C-terminal sequences contribute to the generation of distinct polytene protein binding profiles. Additionally, the lack of co-localisation between MLL fusion proteins and PC or TRX suggests that MLL fusion proteins may not require other PcG/ trxG members for their functions.

The finding that MLL also cannot interact with Pc, as previously shown for TRX (Kuzin et al., 1994; Chinwalla et al., 1995), suggests that in this case, the possibility that the N-terminus of MLL prevents MLL incorporation into TRX complexes, is more appreciable. The lack of extensive co-localisation between MLL and TRX may be expected since only MLL or TRX would be able to incorporate into complexes. The sites, which illustrate MLL and TRX co-localisation may simply reflect MLL and TRX complexes bound to distinct chromatin sites at a higher resolution. Genetic experiments were designed at asking whether MLL can functionally replace TRX during *Drosophila* development. The result that no

rescue of single or of double *trx* mutants by MLL reinforces the double staining experiments between MLL and TRX or Pc. It has been reported that M33, the mouse homologue of Pc, can rescue *pc* -/- mutants, although the rescue was limited to an intermediate level (Muller et al., 1995). The fact that limited conservation between TRX and MLL exists (Chapter 2) accentuates the worthiness that Pc and M33 possess each only two conserved domains, the chromodomain and a stretch of 30 amino acids at their C-terminus (Müller et al, 1995). The later domain has only been found in Pc and M33, and therefore, may facilitate their exchangeability. Taken together, the N-terminus of MLL, which shares limited sequence homology with TRX, may be the primary reason for why MLL cannot functionally exchange with TRX.

Finally, the observation that reduction or absence of a functional *trx* level does not modulate the lethal phenotype induced by MLL-AF9 and MLL-AF4 suggests several implications. First, the lethal phenotype appears to not involve a direct TRX gain-of-function activity. If so, it would be expected that reduction of functional *trx* level should have enhanced MLL fusion protein associated fly viability. Second, the dominant activities relayed by the MLL fusion proteins may inhibit downstream, likely architectural proteins, which may be regulated by TRX at late development and which are necessary for DNA replication or for transcription activation of essential target genes. In this case, lack of functional *trx* would not impact the interference associated with MLL fusion proteins. If anything, an enhancement in the severity of the lethal phenotype is expected. Third, MLL fusion proteins may have a more direct effect on TRX itself by preventing TRX from incorporating into complexes required for its function. This scenario may not be the case since TRX binding patterns seemed intact in MLL-AF9 and in MLL-AF4 flies. However, it is conceivable that TRX alone is required for a specific function necessary for cell proliferation. In this case, MLL fusion proteins would directly prevent TRX from acting, and thus loss of *trx* function would mimic the dominant negative activity by MLL-AF9 and MLL-AF4. Finally, the absence of an effect by TRX on MLL fusion protein-induced lethality may simply reflect a non-existent participation by TRX. Alternatively, the limitation of the experimental set-up may prevent the observation of a partial alleviation of the lethal phenotype by reduction of TRX (such as if viability is restored minimally at the cellular level). The isolation of MLL-AF4 and of MLL-AF9

complexes will be required not only to further understand how MLL fusion proteins derail cell cycle control at the molecular and at the mechanistic levels, but also to unravel more precisely the relation between MLL fusion proteins and TRX during late *Drosophila* development.

CHAPTER 6

Overall Discussion and Perspectives

6.1 Insights into the mode of action for MLL fusion leukaemic proteins.

The use of *Drosophila* to study the direct actions associated with aberrant MLL proteins has unveiled a striking effect on fly viability by these protein fusions. As discussed in Chapter 2, MLL-AF9 and MLL-AF4 specifically induced late larval lethality. Unlike cell culture studies, the results reflected the effects conferred directly by MLL-AF9 and MLL-AF4 in an *in vivo* multi-cellular, chromatin environment. Analyses of animals expressing MLL-AF4 and MLL-AF9 revealed that only the presence of the fusion proteins was not sufficient to cause leukaemia as defined by over-proliferation of blood cells. This probably reflects the necessity for additional mutations, which is precluded by the lethality induced by MLL-AF9 and MLL-AF4. This is consistent with studies of mice harbouring the MLL-AF9 transgene, which develop leukaemia with a latency of 4-12 months (Corral et al., 1996). The monoclonality of these tumours indicated that additional mutations must occur before the appearance of leukaemia (Corral et al., 1996; Dobson et al., 1999).

Furthermore, the *Drosophila* system has addressed the ambiguity involved in the role MLL-AF4 and MLL-AF9 have on apoptosis. As introduced in Section 1.5.4, it is not clear the effect MLL fusion proteins have directly on programmed cell death. By generating cells expressing and not expressing MLL fusion proteins within the same time in development, a correlation between MLL fusion protein expression and apoptosis was unfounded. Thus, it is likely that the apoptosis observed in the mammalian system is an indirect consequence of MLL fusion proteins.

The finding that in *Drosophila* chromatin architecture is altered by MLL fusion proteins substantiates the previous notion that MLL fusion proteins somehow abrogate MLL function. In this respect, possibly, MLL fusion proteins mimic partial loss-of-function mutations for *trx* and *Mll*. Since *trx* and *Mll* act at the chromatin level, modulation of functional TRX/ MLL protein levels conceivably results directly in improper chromatin structures. As discussed previously in Chapter 4, loss of normal *trx* is associated with a striking accumulation of interphase and "prophase-like" nuclei. This phenomenon can be explained by the requirement of *trx* for the proper formation of higher order chromatin structure, prior to the onset of S phase (see Section 4.3.1). In this scenario, lack of functional

trx may disrupt proper chromatin formation of origins recognised by the pre-replication complex (pre-RC), and thus, prevent accessibility of the DNA replication machinery to origins (Fig. 6.1A). In the leukaemic case, data from flies expressing MLL-AF9 and MLL-AF4 indicate that MLL fusion proteins elicit the actions of histone modifying enzymes to alter higher order chromatin structures (see below). The fact that only MLL-AF9 generated aberrant mitotic figures and exhibited faster replicating cells, would suggest that MLL-AF9 creates a more favourable DNA replication environment, by perhaps stabilising higher order chromatin structures, comprising the origins. This chromatin context would enhance pre-RC accessibility to origins, and consequently, a higher initiation of DNA replication occurs, or would reduce G2 phase, as reflected by the number of cells positive for BrdU mitotic figures (Fig 6.1B). The facts that the AT hooks at the N-terminus of MLL remain within the fusion protein and that these AT hooks have been implicated to interact with non-histone HMGs, suggest that MLL-AF9 may interefere with HMGs to create this chromatin context (Zhao et al., 1993; Khadake and Rao, 1997). However, since MLL-AF4 did not exhibit any significant chromosomal chromatin defect or affect DNA replication, this level of chromatin remodelling is minimal, if non-existent. Thus, it is more likely that the effect on DNA replication and on chromosomal abnormalities by MLL-AF9 is a consequence of modulated TRX function and is specific for this fusion protein. Notably, it is not excluded that MLL-AF9 induced lethality may also be independent of TRX function.

The disparity between MLL-AF4 and MLL-AF9 on DNA replication underscores the importance of the C-terminal partners and suggests that (i) the sole disruption of TRX/ MLL function is not sufficient to abrogate cell homeostasis and (ii) that the C-terminal sequences of MLL fusion proteins specify additional pathways, which are targeted by the chimeras. The AF9 C-terminal sequence may have a role in modulating DNA replication, whereas AF4 may be functionally responsible for regulating cell growth. The possibility that *trx* may regulate gene expression of components within the RAS/MAPK and/or PI3/PKB pathways, for example, by modulating *Hox* gene activity, is indicated by late *trx* lethal mutants, which display growth defect phenotypes similar to signal transduction mutants (Breen, 1999).

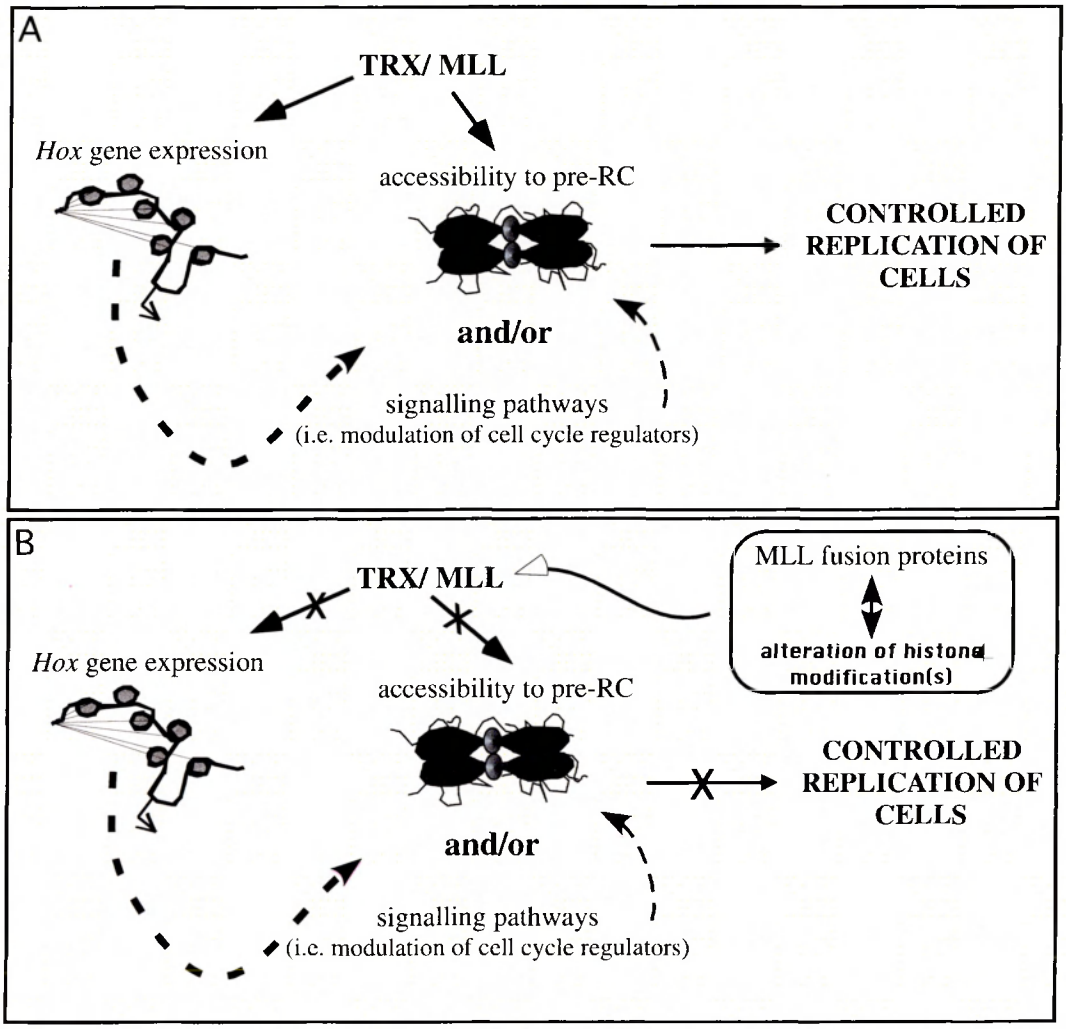


Figure 6.1. Mode of action for MLL fusion leukaemic proteins. The evidence presented in this thesis demonstrates that TRX is required for the completion of late development in *Drosophila*. Particularly, this novel function is distinct from TRX's role as a maintenance factor in early embryogenesis, although similar mechanisms of epigenetic regulation are likely employed by TRX to regulate proper chromatin formation. This finding may account for the extensive number of mutated PcG and trxG members implicated in cancer, a process that occurs late in development. The model presented here speculates that TRX is required to ensure proper higher order chromatin structures necessary for pre-replication complex (pre-RC) binding. Under this condition, DNA replication is possible. In the case for MLL-AF9, MLL-AF9 appears to enhance the stability of higher order chromatin structures dependent on TRX, facilitating the accessibility of origins to pre-RC. In this scenario, DNA replication is favoured and not inhibited. The acceleration of DNA replication facilitates accumulation of secondary mutations, which would be necessary for cell transformation. MLL-AF4, on the other hand, does not exhibit aberrant mitotic figures, although rate of cell cycle divisions was observed to be elevated as in MLL-AF9. The fact that pairing defect on polytenes exists in MLL-AF4, suggests that MLL-AF4 also affects chromatin integrity, apparently at a milder level than MLL-AF9. The alteration in H3K9 methylation pattern supports a role for MLL fusion proteins in modulating chromatin architecture and reinforces the importance of the histone code for cell homeostasis. An effect on the expression of *Hox* genes by *trx* and consequently MLL fusion proteins, can not be excluded as an alternative and intriguing venue for deregulation. In this case, the lack or enhancement of replicating cells would be an indirect consequence of aberrant *Hox* gene expression. In addition, MLL fusion proteins may compromise other aspects controlling cell cycle progression, which exclude the DNA replication machinery. The model proposed centers around the lack of proper chromatin context, and considers the effects of abnormal chromatin conformations on cell functions, such as DNA replication.

6.2 PcG/trxG members are important for the allowance of cell replication.

As discussed above, lack of replicating cells was observed in late *trx* mutants. In addition to its role in chromatin structure formation, conceivably, *trx* may also have a more direct influence on the replication machinery to halt the cell cycle. The connection between proteins required for mitosis and PcG members have already been reported (Lupo et al., 2001). In the study, it was shown that *barren*, a non-SMC (structural maintenance of chromosomes) protein likely associated with condensin, was found to interact with polyhomeotic (Ph). Interestingly, mutants for *ph* and *barren* both exhibit chromosome segregation defects. A more direct evidence of a PcG member interacting with DNA replication factors includes the interaction between cramped (Crm) and PCNA, which enables DNA polymerases to be processive (Yamamoto et al., 1997). In addition, the recent report that in *orc2* mutants, HP1 localisation is perturbed, demonstrates that DNA replication factors, such as ORC2, are closely linked to chromatin regulators, such as HP1 (Pak et al., 1997; Huang et al., 1998). In this respect, it has been suggested that in addition to its general role in DNA replication, ORC is required for *Drosophila* heterochromatin assembly later in embryonic development (Huang et al., 1998; Shareef et al., 2001). Reciprocally, lack of HP1 has been shown to induce telomeric fusion which may have a consequence on the ability of the DNA replication machinery to copy the genome, and thus lead to lethality at late *Drosophila* stages (Fanti et al., 1998). Intriguily, *Drosophila AF10* (the human homologue is a C-terminal partner of an MLL leukaemic fusion protein) was found to associate with HP1 (Linder et al., 2001). This association raises the possibility that the abrogation of the AF10 physiological function by MLL-AF10 leads to release of silenced target genes or that MLL-AF10 recruits HP1-mediated silencing to MLL target genes, which should be kept active.

Importantly, the modulation of *Hox* gene expression cannot be excluded as a possible venue through which *trx* regulates cell proliferation and/ or DNA replication. This possibility becomes more appreciable considering that *Mll* +/- mice displayed retarded growth and reduced B-cell population (Yu et al., 1995). Furthermore, in mammals several *Hox* genes, such as *HoxA9* and *HoxA10*, have been shown to influence proliferation of myeloid progenitors, and

significantly, have induced acute myeloid leukaemia (Nakamura et al., 1996; Bjornsson et al., 2001; Buske et al., 2001).

6.3 MLL-AF4 and MLL-AF9 alter methylation of H3K9.

Although MLL-AF4 and MLL-AF9 appear to target distinct pathways to cause larval to pupal lethality, probably as a function of their C-terminal sequences, MLL-AF4 and MLL-AF9 were found to alter the pattern of methylated H3K9. This result has special significance because it implies that MLL-AF4 and MLL-AF9, and thus possibly all MLL fusion proteins, may employ similar mechanisms to derail their associated pathways. In addition, the importance of the histone code as a regulatory mechanism is reinforced. Modifications in chromatin structure and consequently in DNA replication and cell proliferation, may underlie the leukemogenic effects of MLL fusion proteins. Interestingly, PML-RAR, an oncogenic protein associated with leukaemia, was recently reported to induce gene hypermethylation and silencing of target promoters by recruiting DNA methyltransferases (Di Croce et al., 2002). DNA methylation has been shown to be dependent on histone methylation of H3K9 (Tamaru and Selker, 2001), and therefore, methylation of H3K9 may also be affected by PML-RAR, as shown here with MLL-AF9 and MLL-AF4. The recent demonstration that lack of hSUVAR39 activity induces defects in chromosome segregation and condensation is also clearly reminiscent of the effects described for *trx* and MLL fusion proteins (Peters et al., 2001). This suggests that epigenetic proteins have a greater influence on the stability of chromatin throughout the cell cycle, and that chromatin context is likely to directly influence cell proliferation as shown by the silencing of the cyclin E promoter by the recruitment of the SUVAR39H1-HP1 association by Rb (Nielsen et al., 2001). Indeed, the misregulation of the epigenetic integrity of chromosomal information might play a more important role in the onset of cancer than previously considered. In this respect, "epigenetic crosstalk" has been termed to describe the interplay by several chromatin regulators to establish correct epigenetic chromatin contexts (Ben-Porath and Cedar, 2001).

6.4 Epigenetic regulators have a more prominent role in controlling the cell cycle.

Accumulation of data linking epigenetic regulation with cell cycle control over the recent years has clearly demonstrated the importance of epigenetic mechanisms in cell homeostasis. Epigenetic regulators are classically illustrated as modifiers of chromatin structure maintaining gene expression pattern during early embryogenesis. The finding that key elements ("nodal points", see Section 1.1) contributing to the decision of whether a cell survives or dies, have been epigenetically modified, has uncovered a new pathway through which epigenetics function. A precedent in this respect is the well-established connection between Bmi-1, a PcG mouse homologue of Posterior sex combs (Psc), and ink4a-Arf, a cell cycle control gene. The work presented in this thesis has linked TRX mediated higher order chromatin structures with yet another vital pathway, DNA replication. The implication of these results offers DNA replication as a pathway through which oncogenes may target, with the consequence that additional mutations required for completion of cancer formation is facilitated. In addition, aberrant DNA replication may have a more direct effect on the Rb-E2F proliferative control pathway, since it has been shown that dRb (*Drosophila* Rb) and dE2F (*Drosophila* E2F) complexes appear to regulate origins of DNA replication and that dRb-dE2F interacts with ORC1 and ORC2 (Bosco et al., 2001; Cayirlioglu and Duronio, 2001). The importance of chromatin context in DNA replication is exemplified by the study demonstrating that positions of nucleosomes adjacent to origins by ORC is essential for initiation of replication (Lipford and Bell, 2001).

The work presented here introduced *Drosophila melanogaster* as an additional model system to study the functions directly associated with MLL fusion proteins. The use of *Drosophila* offers a flexible system through which mechanistic questions can now be clarified. Of great interest will be the elucidation of the components which are directly associated with MLL-AF4 and MLL-AF9. Are components of the DNA replication machinery targeted directly by MLL-AF9? Do MLL fusion proteins interact with mutants for other chromatin regulator proteins, such as HP1 or SUVAR39, to partially alleviate their associated lethality? Questions such as these are easier to answer in *Drosophila* since propagation of flies is not

limited, and a large repertoire of mutants for several mitotic genes is available. In addition, a more profound characterisation between *trx* and MLL fusion proteins will be required to define precisely to what extent the lack of *trx* (and thus, *Mll*) function and novel activities contributed by the C-terminal partner contribute to leukemogenesis. Already, MLL-AF9 and MLL-AF4 transgenic flies indicate that the C-terminal partner is crucial for the manifestation of the lethal phenotype and for the fusion protein's specificity in its interference of different pathways. Identification of these pathways through which MLL-AF9 and MLL-AF4 act in *Drosophila*, may also reveal the physiological roles of AF9 and AF4 in mammals, which to date are unknown. By subjecting staged mRNA isolated from MLL, MLL-AF9, and MLL-AF4 expressing animals onto microarrays, results would identify potential target elements modulated by the transgenic proteins. It is clear that *Drosophila* will uncover novel pathways through which MLL-AF4 and MLL-AF9 act. If the behaviour of MLL fusion proteins observed in the fly, remains conserved in mammals, a greater understanding of the role MLL and its C-terminal partners play in leukemogenesis will be achieved.

CHAPTER 7

Materials & Methods

7.1 Materials

7.1.1 Fly stocks

The following stocks were obtained from Bloomington, Indiana, USA, unless otherwise noted. Stocks were raised on standard fly food (10 L water, 80 g agar, 180 g dry yeast, 100 g soya-flour, 220 g honey, 800 g cornmeal, 24 g nipagin (methyl-4-hydroxybensoate; Merck), 62.5 ml propionic acid (Sigma)) at 18 °C or 25 °C with 60-70% relative humidity.

Fly Stock	Obtained from
Wild-type Canton S	P. Becker (Adolf-Butenandt-Institut, Universität Munchen)
w^{1118}	—
trx^{JY16}	T. Breen (Southern Illinois University, US)
trx^{E3}	T. Breen (Southern Illinois University, US)
trx^{B11}	—
B(2;3)/ hs-P[lethal]	G.Merdes (ZMBH, University of Heidelberg, Germany)
armadillo Gal4/ CyO	—
en2.4Gal4/ SM5, Cy	—
GawB/ CyO	—
Act5c-Gal4/ CyO	—
hs-FLP	—
Ay-Gal4 [Act5c-FRT-yellow-FRT-Gal4]	—
daughterless-Gal4	J.Dura (CNRS, Montpellier, France)
V2	A.Brand (Wellcome/CRC Institute, Cambridge, UK)
V37	A.Brand (Wellcome/CRC Institute, Cambridge, UK)
MZ1580	A.Brand (Wellcome/CRC Institute, Cambridge, UK)
gcm/ CyO	F.Sauer (ZMBH, University of Heidelberg, Germany)
elav Gal4	G. Merdes (ZMBH, University of Heidelberg, Germany)
p52A/ CyO	G. Merdes (ZMBH, University of Heidelberg, Germany)

7.1.2 Antibodies

Antigen	Source	Obtained from
FLAG	mouse	Sigma
Phospho-H3	rabbit	Upstate Biotech
TRX	rabbit	this work
Pc	mouse	A. Franke (ZMBH)
BrdU	rat	Harlan Sera Laboratories
MLL (#169)	rabbit	E. Canaani (Weizmann Institute, Israel)
AF4	mouse	E.Canaani (Weizmann Institute, Israel)
me-H3K9	rabbit	T. Jenuwein (Research Institute of Molecular Pathology, Austria)
α-rabbit Cy3	goat	Dianova
α-mouse Alexa 488	goat	Molecular Probes, Inc
α-rat Cy3	goat	Dianova
α-rabbit HRP	goat	Amersham Life Sciences
α-Fluorescine	rabbit	TUNEL assay kit (Roche)

7.1.3 Bacterial strains

The following bacterial strains were used to clone appropriate constructs or to express proteins as indicated in the Methods section.

E. coli strains	Genotype	Practical Use
C600	*thi-1, thr-1, leuB6, lacY1, ton A21, sup E44, mcrA*	TRPE-TRX protein expression
BL21 (DE3)	F^-, ompT, $hsdS_B$, (r_{B-}, m_{B-}), dcm, gal, λ{DE3}	GST-HIS-TRX protein expression
DH5-α	F'/endA1, hsdR17($r_k^- m_k^+$), supE44, thi-1, recA1, gyrA, (Nal^r), relA1, Δ(lacZYA-argF)$_{U169}$ (π80lacZΔM15)	All pUAST cloning
JC8679	F^- *thr-1 leuB-6 phi-1 lacY1 galk2 ara-14 xyl-5 mtl-1 proA2 his-4 argE3 rpsL31 tsx-33 supE44 recB21 recC22 sbcA23 his-328* lambda$^-$	ET cloning

7.1.4 Plasmid vectors

The pATH III vector (Koerner et al., 1991), harbouring the 2388-2674 residues of TRX, was a gift from P. Harte (Case Western Reserve University, Cleveland, OH). TRPE-TRX fusion protein expressed from the pATH III vector was used to immunise rabbits for antibody production. The subcloning of the TRX sequence into the pGEX-4T3 vector (Pharmacia Biotech) allowed purification of specific TRX antibody pools. Both the pATH III and the pGEX-4T3 vectors are ampicillin resistant.

pUAST vector consists of a series of 5 Gal4 binding sites, juxtaposed to the hsp70 minimal promoter, followed by the multiple cloning site and the terminal sequence of SV40, and is selected with ampicillin (Brand and Perrimon, 1993). MLL, MLL-AF9, and MLL-AF4 cDNA sequences were cloned into pUAST for generation of transgenic lines. Detailed description is found in the "DNA Constructs" section in Methods.

7.2. Methods

7.2.1 Generation of affinity pure TRX antibodies

Expression and isolation of TRPE-TRX protein for antibody production.

The TRX residues, spanning 2388-2674 of the TRX sequence, was expressed as a fusion protein with TRPE. The pATH III TRPE-TRX vector was transformed into the expression *E.coli* C600 strain. Colonies were picked and grown overnight in standard LB medium containing 100 μg/ml ampicillin. Overnight cultures were diluted 1:2 in fresh M9 medium (0.5% Casamino acids, 10μg/ml thiamine) supplemented with tryptophan (20μg/ml) and with ampicillin (100 μg/ml) to a final volume of 10 ml. Cells were allowed to grow with vigorous shaking at 37 °C until an O.D. of 0.2-0.4 was reached. The 10 ml culture was then poured into 100 ml M9 medium supplemented only with ampicillin, incubated at 37 °C for 1.5 hours, and subsequently induced with IAA (indoleacrylic acid; 2.5 mg/ml in 95% ethanol) at

10 μg/ml final concentration. After an additional growth of 4 hours with vigorous shaking at 37 °C, cells were harvested in two 50 ml Falcons by centrifugation at 3000 g for 5 minutes.

Extraction of the induced fusion protein was performed as follows. The harvested cells were washed in 50 ml 10 mM Tris pH 7.5, were pelleted again, and were resuspended in 20 ml 10 mM Tris pH 7.5, 5 mM EDTA. To lyse the cells, lysozyme at a concentration of 3 mg/ml was added to the resuspended solution, and the mixture was incubated on ice for 2 hours. 1.4 ml of 5 M NaCl and 1.5 ml of 10% NP40 was added to minimise the number of aggregates, while sonication for 30 s at 350 W disrupted genomic DNA. The processed solution was centrifuged for 10 minutes at 9000 g. The soluble fraction was saved for later analysis on SDS-PAGE, while the pellet was further washed with 20 ml of 10 mM Tris-HCl pH 7.5, 1 M NaCl and with 20 ml of 10 mM Tris-Cl pH 7.5, and was finally resuspended in 1 ml 10 mM Tris-HCl pH 7.5. Both soluble and insoluble fractions were analysed on 10% SDS-PAGE protein gels. The remaining solutions were aliquoted and immediately stored at -80 °C.

Purification of insoluble TRPE-TRX via electrophoresis.

To induced frozen pellets stored at -80 °C, large scale 10% SDS-PAGE electrophoresis was performed using BioRad PROTEAN II. Approximately 300 μl of protein pellet was loaded onto one gel, and the gel was run overnight for good protein separation. For visualisation of the proteins, the SDS-PAGE gel was incubated on ice for 30 minutes to 1 hour in ice-cold 250 mM KCl solution, previously kept on ice overnight. KCl allows the precipitation of proteins in gel at 0 °C; the precipitated proteins appear white in a black background. The desired protein band was excised, and diced to fine pieces to allow efficient recovery of the protein. The gel pieces were then placed on one side of an electrophoresis cup (Colora) filled with eluting buffer (12.5 mM Tris base, 12.5 mM glycine, 10 mM SDS), and elution of protein from the gel pieces was performed for 1.5 hours at 430 V, with occassional agitation every 15-30 minutes. As shown in Figure 7.1, protein migration is driven by electrostatic forces as the negative-charged, SDS coated protein, is attracted to the positive electrode. Around 200 μl of eluted protein was recovered from the positive side of the

electrophoresis cup. 200 µl of PBS was used to wash the chamber and was added to the original eluted protein solution. The protein was then dialysed overnight at 4 °C with stirring against 10 mM NH_4HCO_3/ 10 mM SDS buffer using three changes over 24 hours. The dialysed protein solution was stored at -80 °C. A 10 µl aliquot of this protein solution was run on 10% SDS-PAGE gel to determine the extent of protein degradation, while protein concentration was determined by comparing the Coomassie stained band intensity against a series of known quantities of BSA. A 1:1 mixture of 250 µg of the eluted TRPE-TRX protein and of Freund's Adjuvant (Sigma) was injected into rabbits for antibody production. The rabbits were boosted six times before bleeding.

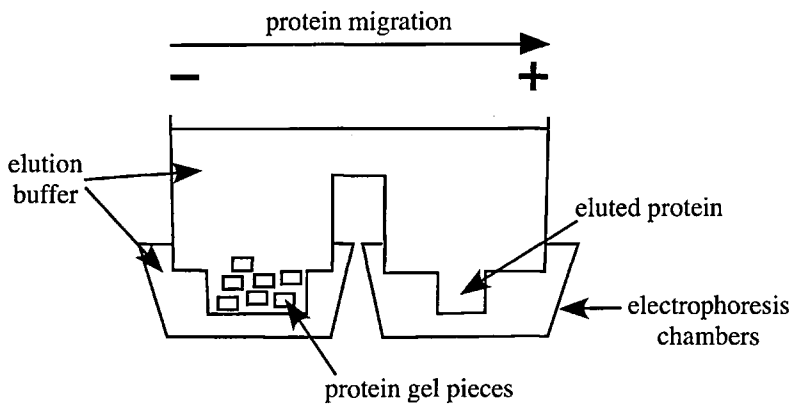


Figure 7.1. Purification of insoluble TRPE-TRX fusion protein. The appropriate band corresponding to the TRPE-TRX fusion protein was excised from a large SDS-PAGE gel. The band containing the fusion protein, was cut to smaller pieces and was transferred to the electrophoresis cup. Elution buffer was filled in both the cup and the electrophoresis chambers to allow the migration of the negatively charged proteins toward the positive electrode. Around 200 µl of eluted protein was recovered.

Purification of rabbit serum for affinity purified TRX antibodies.

Affinity purified TRX antibodies were obtained by purifying the serum over a Protein A column, followed by denaturing and renaturing purification over a nitrocellulose blot. For this purpose, the TRX sequence, corresponding to 2388-2674 residues, was cloned into pGET-4T3 vector to prevent isolation of antibodies against the TRPE portion of the fusion

protein used to immunise rabbits (as described in "Cloning and expression of GST-HIS-TRX fusion protein").

To enrich for IgG antibodies, the serum was subjected to a first round of purification using Affi-Gel Protein A column (Biorad). 5 ml of Affi-Gel Protein A beads were transferred into a 25 ml column capacity (Biorad) and washed with 3 rounds of 20 ml PBS, with 20 ml 0.1 M glycine, pH 2.8, and finally, with sufficient PBS until the pH was neutral. Washing steps were performed at a rate of 3 ml/min.

After equilibration of the Protein A column, 20 ml of undiluted serum was added to the column at a flow rate of 1 ml/min. The serum was allowed to run through the column 3 times. To eliminate unspecific protein binding to the column, PBS was run through the column until the OD_{280} reached less than 0.01. Elution of IgG bound to the column was performed with 20 ml of 0.1 M glycine, pH 2.8. Thirty 1 ml aliquots were recovered, and to each aliquot, 50 µl 1 M Tris pH 8.0 was added to obtain a neutral pH. An OD_{280} profile was obtained for the fractions. The fractions with OD_{280} higher than 1.0 were pooled and stored at -80 °C.

Purified GST-HIS-TRX (as described in "Cloning and expression of GST-HIS-TRX fusion protein") was allowed to bind to nitrocellulose blot at room temperature for 30 minutes. The blot was washed excessively with PBS for 5 minutes, equilibriated with excess 0.1 M glycine, pH 2.8, and neutralised with PBS for 10 minutes. The blot was then incubated with the pooled IgG pure serum (described above) overnight at 4 °C, washed twice with excess PBS for 10 minutes at room temperature, and finally eluted twice with minimal volume (10 ml) of 0.1 M glycine, pH 2.8 for 10 minutes. To the recovered eluted antibodies, sufficient amount of 10x PBS was added to a 1x PBS final concentration. The antibodies were lyophilised overnight and resuspended in 500 µl ddH_20. The resuspended solution was then aliquoted, stored at -20 °C, and tested on polytenes (described in "Antibody staining of polytenes.") and on nuclear extract (described in "Generation of nuclear extract").

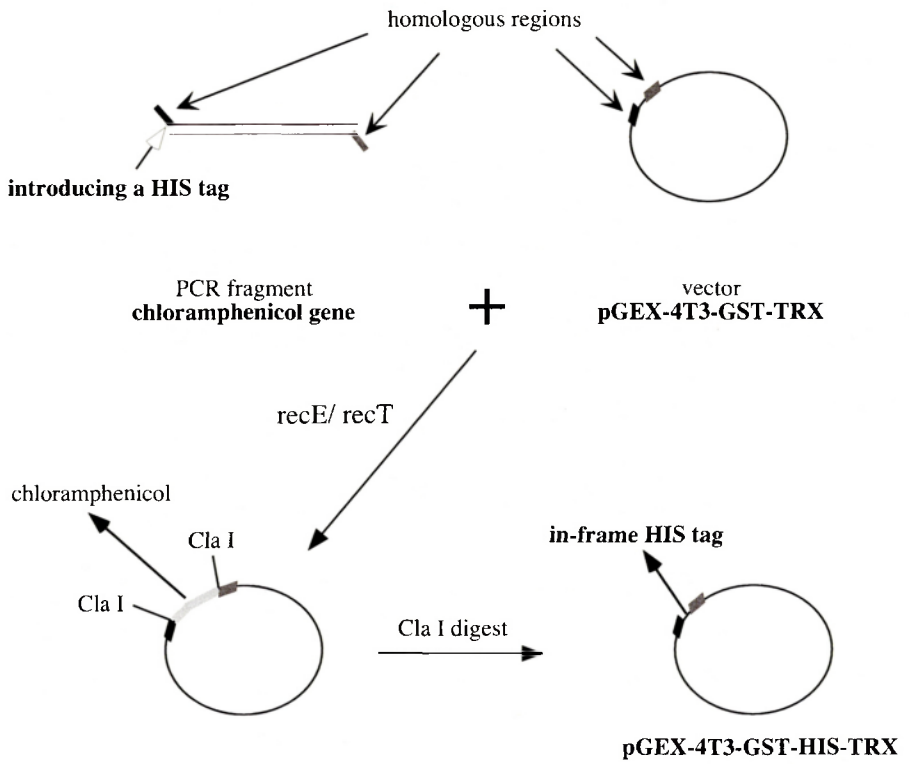
Cloning and expression of GST-HIS-TRX fusion protein.

Resides 2388-2674 of TRX was cloned into the pGEX-4T3 vector by restriction digestion of both pATH III TRPE-TRX and pGEX-4T3 with EcoR I and Sal I. After gel band

elution, isolated bands corresponding to TRX (EcoRI/ Sal I) and to pGEX-4T3 (EcoRI/ Sal I) were ligated overnight to obtain pGEX-4T3-TRX construct. A HIS tag was introduced between the GST and TRX sequences for purification purposes by using the ET cloning method. As shown in Fig. 7.2, ET cloning uses the proteins recE and recT to specifically recombine a linear DNA fragment into a circular plasmid (Zhang et al., 1998). Homology arms, which span the amplified chloramphenicol gene, was designed against the GST sequences of the pGEX-4T3 vector. A ClaI site was introduced to allow excision of the chloramphenicol marker while keeping the GST-HIS-TRX sequences in frame (Fig. 7.2).

The ET reaction was performed as follows (Zhang et al., 1998). Cells constitutively expressing recE and recT (JC8679) were grown to an OD_{280} of 0.4-0.5 in LB medium. The cells were then made electrocompetent by three rounds of harvesting (spins at 7000 rpm for 10 minutes) and resuspending of cells with ice-cold water. Before the final spin, cells were resuspended with ice-cold 10% glycerol/water solution. Final resuspension used the remaining glycerol/water solution within the tubes after removal of excess glycerol/water. Co-electroporation of 0.3 pmol PCR-amplified chloramphenicol fragment and 0.2 pmol pGET-4T3-TRX into 50 µl electro-competent ET cells (JC8679) was performed using Eppendorf Electroporator 2510 at 2000 V. Cells were recovered for 1.5 hours at 37 °C, and correct transformants were selected on LB plates supplemented with ampicillin (100 µg/ml) and chloramphenicol (12.5 µg/ml).

A.



B.

5' Primer: 5' TAA TGT GTG GAA TTG TGA GCG GAT AAC AAT TTC ACA CAG GAA ACA GTA TTC ATG *atc acc atc acc atc ac***a tcg at***ta aat aaa tcc tgg tgt ccc tgt* 3'

3' Primer: 5' TCG AGT GGG TTG CAC AAG GCC CTT AAT TTT CCA ATA ACC TAG TAT AGG GGA **atc gat** *tta cgc ccc gcc ctg cca ctc atc g* 3'

Figure 7.2. Modification of pGEX-4T3-TRX by the ET cloning method. (A) recE and recT allow faithful recombination between two pieces of DNA at a desired location via homologous recombination (reviewed in Muyrers et al., 2001). ET cloning was used to introduce a HIS tag between the GST and the TRX protein sequences. Chloramphenicol was used as a selection marker, and was designed to be excised by Cla I digestion. (B) Primers used in the ET cloning reaction. Capital letters: 5' GST sequence of pGEX-4T3, 3' TRX sequence corresponding to 2388-2404 residues; bold letters: Cla I site; italicised letters: homologous sequence against the chloramphenicol gene used for PCR.

GST-HIS-TRX was expressed by treating cells with IPTG (isopropyl-β-D-thiogalactopyranoside). Cells, diluted 1:10 from overnight cultures, were growth for 2 hours at 37 °C. To the growing cultures, IPTG was added to the final concentration of 1 mM. Cultures were incubated at 37 °C with vigorous shaking for an additional 3 hours. Cells were harvested by centrifugation at 5000 g for 30 minutes.

To purify the insoluble GST-HIS-TRX protein, pellets were resuspended in 50 mM Tris pH 8.0, 1 mg/ml lysozyme (5 ml buffer per 30 ml culture), and incubated for 30 minutes at 30 °C. The resuspension was equilibriated with Triton-X to a final concentration of 0.1 %, and sonified for 10-15 seconds. The cell extract was spun at 20000 g for 15 minutes, and pellets resuspended in 5 ml Binding buffer (20 mM Tris pH 7.9, 0.5 M NaCl, 5 mM imidazole, 8 M Urea) per 30 ml culture. Rotation for 1 hour at room temperature dissolved the pellet containing GST-HIS-TRX. The protein solution was again centrifuged at 39000g for 20 minutes to remove any insoluble debris. The supernatant was added to pre-equilibriated Ni^{2+}-NTA beads (Quiagen), and the resulting slurry solution was rotated for 1-2 hours at room temperature. The bead-protein mixture was poured into a column. The column was washed with 10 column volumes of Binding buffer and with enough Wash buffer (20 mM Tris pH 7.9, 0.5 M NaCl, 60 mM imidazole, 8 M Urea) until OD_{280} was less than 0.01. GST-HIS-TRX protein was eluted with a solution of 20 mM Tris pH 7.9, 0.5 M NaCl, 1 M imidazole, 8 M Urea. The eluted protein was analysed on 10% SDS-PAGE gel and used for affinity antibody purification as described above.

Generation of nuclear extract

2 g of dechorionated embryos from overnight lays was transferred into a glass homogeniser containing 8 ml of buffer B (15 mM Hepes pH 7.6, 10 mM KCl, 5 mM Mg Cl_2, 0.1. mM EDta, 0.5 mM EGTA, 1 mM PMSF, 1 mM DTT, 2 μg/ml leupeptin, pepstatin, aprotinin). The embryos were homogenised with Pestle A, and filtered through Miracloth into 30 ml Corex tubes. The cloth was washed with 5 ml buffer B, and the resulting mixture centrifuged at 2000 g for 10 minutes at 4 °C. The pellet was resuspended in 4 ml buffer B and layered on 1:1 v/ v cushion of buffer B-0.8 M sucrose. After spinning for 10 minutes at

10,000 g using a swing-out rotor, the supernatant was discarded, and the pellet resuspended in 2 ml of buffer B-150 mM KCl. 220 µl of 4 M ammonium-sulphate, pH 7.6, was added, and the mixture was incubated on ice for 15 minutes. The solution was spun for 1 hour at 33,000 g using Beckman SW55Ti rotor. The supernatant was recovered, and proteins were precipitated by adding 0.3 g/ml ammonium sulphate. After 15 minutes spin at 15,000 g at 4 °C, the nuclear protein pellet was resuspended in 300 µl Buffer C (25 mM Hepes pH 7.6, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 10% glycerol).

7.2.2 BLAST analyses for *Drosophila* homologues of the human AF9 and the human AF4 proteins.

Full-length protein sequence of the human AF9 (Accession Number (Pubmed): L13744) and the human AF4 (Accession Number (Pubmed): L13773) proteins were obtained from NCI Pubmed database. Protein sequences were submitted for BLASP analysis against the predicted protein sequences in the available database from the Berkley Genome Sequence Project. Significant matches (i.e. lowest probability against random sequence analysis P(N)) indicated that homologues of AF9 and AF4 exist in *Drosophila melanogaster* (see Figure 2.2).

7.2.3 Generation of transgenic fly lines.

DNA constructs

The appropriate cDNA constructs were cloned into pUAST through a series of PCR amplification and ligation steps. Correct cloning of sequences was monitored after each PCR/ligation round by sequencing. The full-length MLL cDNA was cloned into the EcoRI site of the pUAST vector. The fusion MLL-AF9 construct consist of the first 4086 nucleotides from the MLL cDNA juxtaposed to 1432-1704 nucleotide sequence from the AF9 cDNA. The MLL-AF9 fusion construct was cloned into the EcoRI site of pUAST. Additionally, both the MLL and MLL-AF9 construct contains a FLAG epitope (GAC TAC AAG GAC GAC GAT GAC AAG) 3' to the end of the cDNA sequence. The MLL-AF4 construct

includes the first 4218 nucleotides of the MLL cDNA sequence fused in frame to the 1041-3633 nucleotides from the AF4 cDNA sequence. The MLL-AF4 construct was cloned into the Xho I and Xba I sites of pUAST and can be released by double digestion with Xho I and Nhe I.

Transformation of w^{1118} with pUAST constructs.

The appropriate pUAST constructs (300 ng/µl, described in "DNA Constructs") were coinjected into w^{1118} embryos with the helper DNA pUChsπΔ2-3 (100 ng/µl). Embryos were recovered from 30 minutes egg lays at 25 °C. The embryos were transferred to 18 °C, were dechorionised with a 1:5 dilution of bleach (14%)-water solution, and then were washed extensively with water until bleach odor was no longer detectable. Around 70-100 embryos were lined on 16% agar plates and were transferred onto a double-sided sticky tape on a coverslip. The embryos were dehydrated in a closed chamber containing Silica gel for 7 minutes and then covered with Voltalef 10 S oil (Lehmann & Voss & Co.). Microinjection of the pUAST/helper DNA solution into embryos was performed using the Eppendorf FemtoJet microinjector. Larvae from injected embryos were collected into standard fly food and allowed to develop. Eclosed flies were crossed to w^{1118} virgins or males; progenies were then scored for pigmented eyes. At least 5 different independent transformants were kept as stocks, and 2 lines were selected for experiments.

7.2.4 RT-PCR & Western Blot Analysis

RT-PCR (Reverse-Transcription PolyChain Reaction) and Western blot analysis were employed to verify that the desired proteins were expressed when GAL4 was present.

For detection of specific mRNA, 10 mg worth of heads from appropriate transgenic fly lines was attained. Process of tissue samples was performed according to instructions in the Invitrogen's SNAP Total RNA Isolation kit. Primers used for detection of specific mRNA are the following: 5'PRIMER: 5' GTG ACT CTT GGG GAT ACA ACA GCT GTC 3'; 3'PRIMER: 5' GTC CAC CAC GTT CTT CAC AAC ACT GCT 3'. These primers should amplify a 900 bp fragment corresponding to the sequence of 2842-3717 of MLL cDNA.

For detection of appropriate proteins, imaginal discs were isolated from 20 larvae and homogenised in 1xSDS buffer. The extract was then boiled for 10 min, and loaded onto 5% SDS-PAGE gels. The protein contents from the SDS gel was transferred onto nitrocellulose membrane (Hybond^{TM}ECL^{TM}) overnight at 100 mA, using the 0.0037% SDS, 39 mM glycine, 48 mM Tris, 20% methanol Transfer Buffer. Nitrocellulose blots were then processed with α-MLL N-terminal rabbit antibodies overnight at 1:1000 dilution in 0.2% Tween-20/PBS, at 4 °C, followed by 2 times 15 minutes washes with 0.2% Tween-20 /PBS, and incubated with HRP conjugated secondary antibodies against rabbit (1:5000) for 1 hour at room temperature. After washing the pellet 3 times with 0.2% Tween-20/PBS for each 10 minutes, signals were detected according to instructions from ECL^{TM} (Amersham Pharmacia Biotech). Namely, the blot was precisely incubated for 1 minute with a solution of 1:1 volume of solution 1 and of solution 2. Excess liquid was removed, and the processed blot was sealed in plastic to prevent drying out. Films were immediately placed on the sealed blot for signal detection.

7.2.5 Phenotypic analyses of transgenic fly lines with various GAL4 driver lines.

To determine the effect expression of MLL, of MLL-AF9, and of MLL-AF4 has on fly development, virgins from transgenic lines were crossed to males from several Gal4 driver strains. Crosses were kept at 18 °C, 25 °C, or 29 °C. Progenies were then scored for the effect the trangenic proteins have on fly development.

7.2.6 Generation of transgenic lines in a double *trx* mutant background.

To determine if expression of MLL can functionally rescue *trx* mutant phenotypes, appropriate *trx* alleles (trx^{JY16}, trx^{B11}), and transgenic fly lines or Gal4 driver strains, were first balanced over B(2;3), and then crossed to one another to generate a stable stock consisting of the following genotype: transgenic fly line/ Gal4 driver strain; *trx* allele/ B(2;3). Rescue of the embryonic or larval lethality associated with the *trx* mutant background was monitored upon expression of appropriate transgenic proteins with various Gal4 driver lines.

7.2.7 Isolation of the hemolymph and hemocyte counts from *Drosophila* transgenic lines.

Larvae expressing transgenic protein by using the blood Gal4 driver line, MZ1580, was washed in 100% ethanol and dried on filter paper. Using 5c forceps (Sigma), the cuticle was carefully opened to avoid breaking fat bodies. The hemolymph was allowed to accumulate around the larval carcass. Using a 20 µl-micropipettor, the hemolymph, which varies from 0.2-0.5 µl, was collected, and was transferred to a hemocytometer, which had been covered with minerol oil. The coverslip was dropped on top of the sample and cells were counted for six 50 µm x 50 µm (according to the laboratory of S. Govind, City College, New York, NY). The number of hemocytes per µl was determined by using the following formula: the number of hemocytes/ (1/ 0.024 mm^3).

7.2.8 TUNEL assay of imaginal discs

Clones which express MLL, MLL-AF4, or MLL-AF9 were generated by heat-shocking first instar larvae for 1 hour at 37 °C. Larvae were recovered at 25 °C. Imaginal discs from developed third instar larvae were isolated and subjected to the TUNEL assay (In Situ Cell Death Detection Kit, AP from Roche). Briefly, imaginal discs were fixed with 4% PFA/PBS for 20 minutes on ice, were washed 3 times 5 minutes with PBS, and were permeabilised in 0.1% Triton X-100/ 0.1% NaCitrate/PBS. After two times PBS washes, apoptotic cells in imaginal discs were labelled with the TUNEL reaction mix for 1 hour at 37 °C. Again, the labelled imaginal discs were washed 3 times with PBS for 5 minutes. Antibodies against Fluorescine (1:80) were incubated with the imaginal discs overnight at 4 °C. The imaginal discs were washed with PBS, and incubated with a secondary antibody (α-rabbit-Cy3) for signal amplification. After PBS washing, the discs were mounted in Mowiol.

7.2.9 Brain & polytene squashes

Detection of metaphase spreads from brain squashes by DAPI staining.

To detect mitotic figures, two brains were isolated in 0.7% NaCl. The brains were then transfered onto poly-L-lysine (PLL) slides, and incubated for 30 sec in 45% Acetic Acid/PBS followed by a 3 minutes incubation with 60% Acetic Acid/PBS. A freshly cleaned cover slip was gently placed on top of the brains. Between two layers of Whatmann paper, the brains were squashed without moving the cover slip. Slides were immersed in liquid nitrogen, and cover slips flicked off. Subsequently, slides were dehydrated in two consequetive steps with 70% EtOH followed by 100% EtOH for each 3 minutes. To overnight air-dried slides, rehydration in PBS was performed for 2 minutes, followed by 5 minutes DAPI (200 ng/ml) staining. Slides were allowed to drain from excess PBS solution and mounted in Mowiol.

Colchicine and hypotonic treatments of brains.

Immediately after isolation of intact whole brains, brains were either treated with 10^{-3} M colchicine solution dissolved in PBS for 1.5 hours at 25 °C, or were directly processed in the following way. 0.5% citrate solution was used to hypotonic treat the brains for 7 minutes. Colchicine treatment halts cell cycle progression at metaphase while hypotonic treatment separates sister chromosomes.

Phospho-H3 labelling of squashed brain preparations.

Phospho-H3 is used as an indicator for replicating cells, since histone H3 is phosphorylated during mitosis. For staining of brain squashed preparation with histone H3, the brain squash protocol (described in "Detection of metaphase spreads from brain squashes by DAPI staining") was modifed in the following manner. Isolated brains were transferred into a drop of 25% acetic acid/PBS and squashed as described above. The squashed preparation was then incubated for a further 5 minutes. After removal of the cover slip with liquid

hydrogen, slides were washed in PBS for 2 minutes and then fixed in 4% PFA/ 45% acetic acid/ PBS solution for 10 minutes. Subsequently, the slide was washed in 0.1% Triton/ PBS (PBS-T), blocked for 1 hour in fresh 3% BSA/ PBS blocking solution, and incubated overnight with 1:500 dilution of phospho-H3 antibodies. The slide was washed three times with PBS-T for each 5 minutes, and then incubated for 1.5 hours with secondary antibodies at a dilution of 1:200 (rabbit Alexa 488). Again, the slides were washed with PBS-T three times at each 5 minutes. DAPI staining and mounting were then performed as described previously.

BrdU labelling of brains *in vitro* and *in vivo*.

At least 4-5 whole brains were dissected from appropriate fly lines, and incubated in 0.1 mg/ml BrdU dissolved in 0.7% NaCl, for 1.5 hours at 25 °C. The brains were then rinsed with PBS and permeabilised for 10 minutes using 0.1% Triton-X/PBS (PBS-T). A 4% paraformaldehyde-PBS solution was used to fix the brains for 20 minutes. After rinsing with PBS-T for 5 minutes, the brains were incubated in 2 N HCl for 30 minutes at room temperature, followed by a 10 minutes PBS-T wash. The brains were blocked with 0.3% BSA in PBS for 30 minutes at room temperature, and then, incubated with 1:2 dilution of α-BrdU rat monoclonal antibodies overnight. Detection for BrdU labelling was performed with α-rat Cy3 secondary antibodies at 1:200 dilution, while nuclei were stained with DAPI (200 ng/ml).

For *in vivo* labelling of brains with BrdU, larvae were fed with a mixture of 1 g *Drosophila* Instant food (Sigma) and of 100 mg BrdU dissolved in 2 ml water. Red food coloring was added to allow detection of ingested third-instar larvae. Brain squash and detection of BrdU were then performed.

Orcein staining of polytenes and brain squashes.

Salivary glands and brains were isolated in 45% acetic acid/PBS. The tissue was transferred to a drop of 1 N HCl and incubated precisely for 1 minute. The tissue was then washed with 50% lactic acid/ 30% acetic acid, was stained for 10 minutes with orcein (2% orcein/ 30% lactic acid/ 30% acetic acid), and was rinsed with 45% acetic acid. A clean

coverslip was then placed on top of the tissue. After brain squashing, the slides were sealed with nail polish.

Antibody staining of polytenes.

Two pairs of salivary glands were isolated in 0.7% NaCl and were transferred onto one poly-L-lysine (PLL) coated slide. The glands were incubated in 45% Acetic Acid / 5% fresh para-formaldehyde / ddH_20 fixative for 10 minutes. Salivary glands were then squashed and were spread by gently moving the cover slip. Slides were immersed in liquid nitrogen, and coverslips flicked off. Slides were washed in PBS twice for 5 minutes, permeabilised for 10 minutes in 1% Triton-X/PBS, blocked in a saturated solution of non-fat milk powder (blocking solution) for 30 minutes, and incubated with appropriate primary antibody dilution (mouse α-Pc 1:40, rabbit α-meH3K9 1:500, rabbit α-TRX 1:10; mouse α-FLAG (Sigma) 1:200) for 1 hour at room temperature, followed by overnight at 4 °C. After washing three times with saturated solution of milk powder, slides were incubated for 1 hour with secondary antibodies (α-rabbit Alexa-488, 1:200; α-mouse Cy3, 1:500) at room temperature. Slides were rinsed in PBS, and washed for 15 minutes in 0.2% NP-40, 0.2% Tween 20-80, 300 mM NaCl, and in 0.2% NP-40, 0.2% Tween 20-80, 400 mM NaCl solutions. If me-H3K9 antibodies were used, an additional 15 minutes washing step was performed with 0.2% NP-40, 0.2% Tween 20-80, 500 mM NaCl solution. Finally, the slides were DAPI (100 ng/μl) stained for 10 minutes and were mounted in Mowiol.

CHAPTER 8

References

Aasland, R., Gibson, T.J. and Stewart, A.F. (1995) The PHD finger: implications for chromatin-mediated transcriptional regulation. *Trends Biochem Sci*, **20**, 56-59.

Adams, M.D., Celniker, S.E., Holt, R.A., Evans, C.A., Gocayne, J.D., Amanatides, P.G., Scherer, S.E., Li, P.W., Hoskins, R.A., Galle, R.F., George, R.A., et al. (2000) The genome sequence of Drosophila melanogaster. *Science*, **287**, 2185-2195.

Adler, H.T., Chinery, R., Wu, D.Y., Kussick, S.J., Payne, J.M., Fornace, A.J., Jr. and Tkachuk, D.C. (1999) Leukemic HRX fusion proteins inhibit GADD34-induced apoptosis and associate with the GADD34 and hSNF5/INI1 proteins. *Mol Cell Biol*, **19**, 7050-7060.

Ajiro, K. (2000) Histone H2B phosphorylation in mammalian apoptotic cells. An association with DNA fragmentation. *J Biol Chem*, **275**, 439-443.

Akasaka, T., Kanno, M., Balling, R., Mieza, M.A., Taniguchi, M. and Koseki, H. (1996) A role for mel-18, a Polycomb group-related vertebrate gene, during theanteroposterior specification of the axial skeleton. *Development*, **122**, 1513-1522.

Akasaka, T., van Lohuizen, M., van der Lugt, N., Mizutani-Koseki, Y., Kanno, M., Taniguchi, M., Vidal, M., Alkema, M., Berns, A. and Koseki, H. (2001) Mice doubly deficient for the Polycomb Group genes Mel18 and Bmi1 reveal synergy and requirement for maintenance but not initiation of Hox gene expression. *Development*, **128**, 1587-1597.

Akhtar, A., Zink, D. and Becker, P.B. (2000) Chromodomains are protein-RNA interaction modules. *Nature*, **407**, 405-409.

Alkema, M.J., Bronk, M., Verhoeven, E., Otte, A., van't Veer, L.J., Berns, A. and van Lohuizen, M. (1997a) Identification of Bmi1-interacting proteins as constituents of a multimeric mammalian polycomb complex. *Genes Dev*, **11**, 226-240.

Alkema, M.J., Jacobs, J., Voncken, J.W., Jenkins, N.A., Copeland, N.G., Satijn, D.P., Otte, A.P., Berns, A. and van Lohuizen, M. (1997b) MPc2, a new murine homolog of the Drosophila polycomb protein is a member of the mouse polycomb transcriptional repressor complex. *J Mol Biol*, **273**, 993-1003.

Andersen, M.K., Christiansen, D.H., Kirchhoff, M. and Pedersen-Bjergaard, J. (2001) Duplication or amplification of chromosome band 11q23, including the unrearranged MLL gene, is a recurrent abnormality in therapy-related MDS and AML, and is closely related to mutation of the TP53 gene and to previous therapy with alkylating agents. *Genes Chromosomes Cancer*, **31**, 33-41.

Armstrong, S.A., Staunton, J.E., Silverman, L.B., Pieters, R., den Boer, M.L., Minden, M.D., Sallan, S.E., Lander, E.S., Golub, T.R. and Korsmeyer, S.J. (2002) MLL translocations specify a distinct gene expression profile that distinguishes a unique leukemia. *Nat Genet*, **30**, 41-47.

Ayton, P., Sneddon, S.F., Palmer, D.B., Rosewell, I.R., Owen, M.J., Young, B., Presley, R. and Subramanian, V. (2001) Truncation of the Mll gene in exon 5 by gene targeting leads to early preimplantation lethality of homozygous embryos. *Genesis*, **30**, 201-212.

Ayton, P.M. and Cleary, M.L. (2001) Molecular mechanisms of leukemogenesis mediated by MLL fusion proteins. *Oncogene*, **20**, 5695-5707.

Baguisi, A., Behboodi, E., Melican, D.T., Pollock, J.S., Destrempes, M.M., Cammuso, C., Williams, J.L., Nims, S.D., Porter, C.A., Midura, P., Palacios, M.J., Ayres, S.L., Denniston, R.S., Hayes, M.L., Ziomek, C.A., Meade, H.M., Godke, R.A., Gavin, W.G., Overstrom, E.W. and Echelard, Y. (1999) Production of goats by somatic cell nuclear transfer. *Nat Biotechnol*, **17**, 456-461.

Bannister, A.J., Zegerman, P., Partridge, J.F., Miska, E.A., Thomas, J.O., Allshire, R.C. and Kouzarides, T. (2001) Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature*, **410**, 120-124.

Bantignies, F., Goodman, R.H. and Smolik, S.M. (2000) Functional interaction between the coactivator Drosophila CREB-binding protein and ASH1, a member of the trithorax group of chromatin modifiers. *Mol Cell Biol*, **20**, 9317-9330.

Bea, S., Tort, F., Pinyol, M., Puig, X., Hernandez, L., Hernandez, S., Fernandez, P.L., van Lohuizen, M., Colomer, D. and Campo, E. (2001) BMI-1 gene amplification and overexpression in hematological malignancies occur mainly in mantle cell lymphomas. *Cancer Res*, **61**, 2409-2412.

Ben-Porath, I. and Cedar, H. (2001) Epigenetic crosstalk. *Mol Cell*, **8**, 933-935.

Bernardoni, R., Vivancos, B. and Giangrande, A. (1997) glide/gcm is expressed and required in the scavenger cell lineage. *Dev Biol*, **191**, 118-130.

Betti, C.J., Villalobos, M.J., Diaz, M.O. and Vaughan, A.T. (2001) Apoptotic triggers initiate translocations within the MLL gene involving the nonhomologous end joining repair system. *Cancer Res*, **61**, 4550-4555.

Bilder, D., Li, M. and Perrimon, N. (2000) Cooperative regulation of cell polarity and growth by Drosophila tumor suppressors. *Science*, **289**, 113-116.

Binari, R. and Perrimon, N. (1994) Stripe-specific regulation of pair-rule genes by hopscotch, a putative Jak family tyrosine kinase in Drosophila. *Genes Dev*, **8**, 300-312.

Birke, M., Schreiner, S., Garcia-Cuellar, M.P., Mahr, K., Titgemeyer, F. and Slany, R.K. (2002) The MT domain of the proto-oncoprotein MLL binds to CpG-containing DNA and discriminates against methylation. *Nucleic Acids Res*, **30**, 958-965.

Bjornsson, J.M., Andersson, E., Lundstrom, P., Larsson, N., Xu, X., Repetowska, E., Humphries, R.K. and Karlsson, S. (2001) Proliferation of primitive myeloid progenitors can be reversibly induced by HOXA10. *Blood*, **98**, 3301-3308.

Blanco, J.G., Dervieux, T., Edick, M.J., Mehta, P.K., Rubnitz, J.E., Shurtleff, S., Raimondi, S.C., Behm, F.G., Pui, C.H. and Relling, M.V. (2001) Molecular emergence of acute myeloid leukemia during treatment for acute lymphoblastic leukemia. *Proc Natl Acad Sci U S A*, **98**, 10338-10343.

Bochar, D.A., Wang, L., Beniya, H., Kinev, A., Xue, Y., Lane, W.S., Wang, W., Kashanchi, F. and Shiekhattar, R. (2000) BRCA1 is associated with a human SWI/SNF-related complex: linking chromatin remodeling to breast cancer. *Cell*, **102**, 257-265.

Boggs, B.A., Cheung, P., Heard, E., Spector, D.L., Chinault, A.C. and Allis, C.D. (2002) Differentially methylated forms of histone H3 show unique association patterns with inactive human X chromosomes. *Nat Genet*, **30**, 73-76.

Boivin, A. and Dura, J.M. (1998) In vivo chromatin accessibility correlates with gene silencing in Drosophila. *Genetics*, **150**, 1539-1549.

Boman HG, Nilsson I, Rasmuson B. (1972) Inducible antibacterial defence system in Drosophila. *Nature*, **237**, 232-5.

Bosco, G., Du, W. and Orr-Weaver, T.L. (2001) DNA replication control through interaction of E2F-RB and the origin recognition complex. *Nat Cell Biol*, **3**, 289-295.
taf/DynaPage.taf?file=/ncb/journal/v283/n283/full/ncb0301_0289.html
taf/DynaPage.taf?file=/ncb/journal/v0303/n0303/abs/ncb0301_0289.html.

Bradbury, E.M. (1992) Reversible histone modifications and the chromosome cell cycle. *Bioessays*, **14**, 9-16.

Brand, A.H. and Perrimon, N. (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development*, **118**, 401-415.

Braunstein, M., Sobel, R.E., Allis, C.D., Turner, B.M. and Broach, J.R. (1996) Efficient transcriptional silencing in Saccharomyces cerevisiae requires a heterochromatin histone acetylation pattern. *Mol Cell Biol*, **16**, 4349-4356.

Breen, T.R. (1999) Mutant alleles of the Drosophila trithorax gene produce common and unusual homeotic and other developmental phenotypes. *Genetics*, **152**, 319-344.

Brehm, A., Miska, E.A., McCance, D.J., Reid, J.L., Bannister, A.J. and Kouzarides, T. (1998) Retinoblastoma protein recruits histone deacetylase to repress transcription. *Nature*, **391**, 597-601.

Breiling, A., Bonte, E., Ferrari, S., Becker, P.B. and Paro, R. (1999) The Drosophila polycomb protein interacts with nucleosomal core particles In vitro via its repression domain. *Mol Cell Biol*, **19**, 8451-8460.

Breiling, A., Turner, B.M., Bianchi, M.E. and Orlando, V. (2001) General transcription factors bind promoters repressed by Polycomb group proteins. *Nature*, **412**, 651-655.

Briggs, S.D., Bryk, M., Strahl, B.D., Cheung, W.L., Davie, J.K., Dent, S.Y., Winston, F. and Allis, C.D. (2001) Histone H3 lysine 4 methylation is mediated by Set1 and required for cell growth and rDNA silencing in Saccharomyces cerevisiae. *Genes Dev*, **15**, 3286-3295.

Brock, H.W. and van Lohuizen, M. (2001) The Polycomb group--no longer an exclusive club? *Curr Opin Genet Dev*, **11**, 175-181.

Brown, J.L., Mucci, D., Whiteley, M., Dirksen, M.L. and Kassis, J.A. (1998) The Drosophila Polycomb group gene pleiohomeotic encodes a DNA binding protein with homology to the transcription factor YY1. *Mol Cell*, **1**, 1057-1064.

Brownell, J.E., Zhou, J., Ranalli, T., Kobayashi, R., Edmondson, D.G., Roth, S.Y. and Allis, C.D. (1996) Tetrahymena histone acetyltransferase A: a homolog to yeast Gcn5p linking histone acetylation to gene activation. *Cell*, **84**, 843-851.

Brzovic, P.S., Rajagopal, P., Hoyt, D.W., King, M.C. and Klevit, R.E. (2001) Structure of a BRCA1-BARD1 heterodimeric RING-RING complex. *Nat Struct Biol*, **8**, 833-837.

Buske, C., Feuring-Buske, M., Antonchuk, J., Rosten, P., Hogge, D.E., Eaves, C.J. and Humphries, R.K. (2001) Overexpression of HOXA10 perturbs human lymphomyelopoiesis in vitro and in vivo. *Blood*, **97**, 2286-2292.

Cairns, B.R., Henry, N.L. and Kornberg, R.D. (1996) TFG/TAF30/ANC1, a component of the yeast SWI/SNF complex that is similar to the leukemogenic proteins ENL and AF-9. *Mol Cell Biol*, **16**, 3308-3316.

Caslini, C., Shilatifard, A., Yang, L. and Hess, J.L. (2000) The amino terminus of the mixed lineage leukemia protein (MLL) promotes cell cycle arrest and monocytic differentiation. *Proc Natl Acad Sci U S A*, **97**, 2797-2802.

Castilla, L.H., Wijmenga, C., Wang, Q., Stacy, T., Speck, N.A., Eckhaus, M., Marin-Padilla, M., Collins, F.S., Wynshaw-Boris, A. and Liu, P.P. (1996) Failure of embryonic hematopoiesis and lethal hemorrhages in mouse embryos heterozygous for a knocked-in leukemia gene CBFB-MYH11. *Cell*, **87**, 687-696.

Cavalli, G. and Paro, R. (1998) The Drosophila Fab-7 chromosomal element conveys epigenetic inheritance during mitosis and meiosis. *Cell*, **93**, 505-518.

Cavalli, G. and Paro, R. (1999) Epigenetic inheritance of active chromatin after removal of the main transactivator. *Science*, **286(5441)**, 955-8.

Cayirlioglu, P., Bonnette, P.C., Dickson, M.R. and Duronio, R.J. (2001) Drosophila E2f2 promotes the conversion from genomic DNA replication to gene amplification in ovarian follicle cells. *Development*, **128**, 5085-5098.

Cayirlioglu, P. and Duronio, R.J. (2001) Cell cycle: Flies teach an old dogma new tricks. *Curr Biol*, **11**, R178-181.

Chadee, D.N., Hendzel, M.J., Tylipski, C.P., Allis, C.D., Bazett-Jones, D.P., Wright, J.A. and Davie, J.R. (1999) Increased Ser-10 phosphorylation of histone H3 in mitogen-stimulated and oncogene-transformed mouse fibroblasts. *J Biol Chem*, **274**, 24914-24920.

Charite, J., de Graaff, W., Consten, D., Reijnen, M.J., Korving, J. and Deschamps, J. (1998) Transducing positional information to the Hox genes: critical interaction of cdx gene products with position-sensitive regulatory elements. *Development*, **125**, 4349-4358.

Chen, D., Ma, H., Hong, H., Koh, S.S., Huang, S.M., Schurter, B.T., Aswad, D.W. and Stallcup, M.R. (1999) Regulation of transcription by a protein methyltransferase. *Science*, **284**, 2174-2177.

Cheung, P., Allis, C.D. and Sassone-Corsi, P. (2000) Signaling to chromatin through histone modifications. *Cell*, **103**, 263-271.

Chinwalla, V., Jane, E.P. and Harte, P.J. (1995) The Drosophila trithorax protein binds to specific chromosomal sites and is co-localized with Polycomb at many sites. *Embo J*, **14**, 2056-2065.

Clayton, A.L., Rose, S., Barratt, M.J. and Mahadevan, L.C. (2000) Phosphoacetylation of histone H3 on c-fos- and c-jun-associated nucleosomes upon gene activation. *Embo J*, **19**, 3714-3726.

Collins, E.C., Pannell, R., Simpson, E.M., Forster, A. and Rabbitts, T.H. (2000) Inter-chromosomal recombination of Mll and Af9 genes mediated by cre- loxP in mouse development. *EMBO Rep*, **1**, 127-132.

Collins, R.T., Furukawa, T., Tanese, N. and Treisman, J.E. (1999) Osa associates with the Brahma chromatin remodeling complex and promotes the activation of some target genes. *Embo J*, **18**, 7029-7040.

Core, N., Bel, S., Gaunt, S.J., Aurrand-Lions, M., Pearce, J., Fisher, A. and Djabali, M. (1997) Altered cellular proliferation and mesoderm patterning in Polycomb-M33- deficient mice. *Development*, **124**, 721-729.

Corral, J., Lavenir, I., Impey, H., Warren, A.J., Forster, A., Larson, T.A., Bell, S., McKenzie, A.N., King, G. and Rabbitts, T.H. (1996) An Mll-AF9 fusion gene made by homologous recombination causes acute leukemia in chimeric mice: a method to create fusion oncogenes. *Cell*, **85**, 853-861.

Cronmiller, C. and Cummings, C.A. (1993) The daughterless gene product in Drosophila is a nuclear protein that is broadly expressed throughout the organism during development. *Mech Dev*, **42**, 159-169.

Crosby, M.A., Miller, C., Alon, T., Watson, K.L., Verrijzer, C.P., Goldman-Levi, R. and Zak, N.B. (1999) The trithorax group gene moira encodes a brahma-associated putative chromatin-remodeling factor in Drosophila melanogaster. *Mol Cell Biol*, **19**, 1159-1170.

Cross, S.H., Meehan, R.R., Nan, X. and Bird, A. (1997) A component of the transcriptional repressor MeCP1 shares a motif with DNA methyltransferase and HRX proteins. *Nat Genet*, **16**, 256-259.

Cui, X., De Vivo, I., Slany, R., Miyamoto, A., Firestein, R. and Cleary, M.L. (1998) Association of SET domain and myotubularin-related proteins modulates growth control. *Nat Genet*, **18**, 331-337.

Dahiya, A., Wong, S., Gonzalo, S., Gavin, M. and Dean, D.C. (2001) Linking the Rb and polycomb pathways. *Mol Cell*, **8**, 557-569.

Dang, D.T. and Perrimon, N. (1992) Use of a yeast site-specific recombinase to generate embryonic mosaics in Drosophila. *Dev Genet*, **13**, 367-375.

De Lorenzo, C., Mechler, B.M. and Bryant, P.J. (1999) What is Drosophila telling us about cancer? *Cancer Metastasis Rev*, **18**, 295-311.

Debernardi, S., Bassini, A., Jones, L.K., Chaplin, T., Linder, B., de Bruijn, D.R., Meese, E. and Young, B.D. (2002) The MLL fusion partner AF10 binds GAS41, a protein that interacts with the human SWI/SNF complex. *Blood*, **99**, 275-281.

DeCamillis, M., Cheng, N.S., Pierre, D. and Brock, H.W. (1992) The polyhomeotic gene of Drosophila encodes a chromatin protein that shares polytene chromosome-binding sites with Polycomb. *Genes Dev*, **6**, 223-232.

Deschamps, J., van den Akker, E., Forlani, S., De Graaff, W., Oosterveen, T., Roelen, B. and Roelfsema, J. (1999) Initiation, establishment and maintenance of Hox gene expression patterns in the mouse. *Int J Dev Biol*, **43**, 635-650.

Dhalluin, C., Carlson, J.E., Zeng, L., He, C., Aggarwal, A.K. and Zhou, M.M. (1999) Structure and ligand of a histone acetyltransferase bromodomain. *Nature*, **399**, 491-496.

Di Croce, L., Raker, V.A., Corsaro, M., Fazi, F., Fanelli, M., Faretta, M., Fuks, F., Lo Coco, F., Kouzarides, T., Nervi, C., Minucci, S. and Pelicci, P.G. (2002) Methyltransferase recruitment and DNA hypermethylation of target promoters by an oncogenic transcription factor. *Science*, **295**, 1079-1082.

DiMartino, J.F., Miller, T., Ayton, P.M., Landewe, T., Hess, J.L., Cleary, M.L. and Shilatifard, A. (2000) A carboxy-terminal domain of ELL is required and sufficient for immortalization of myeloid progenitors by MLL-ELL. *Blood*, **96**, 3887-3893.

Dingwall, A.K., Beek, S.J., McCallum, C.M., Tamkun, J.W., Kalpana, G.V., Goff, S.P. and Scott, M.P. (1995) The Drosophila snr1 and brm proteins are related to yeast SWI/SNF proteins and are components of a large protein complex. *Mol Biol Cell*, **6**, 777-791.

Dobson, C.L., Warren, A.J., Pannell, R., Forster, A., Lavenir, I., Corral, J., Smith, A.J. and Rabbitts, T.H. (1999) The mll-AF9 gene fusion in mice controls myeloproliferation and specifies acute myeloid leukaemogenesis. *Embo J*, **18**, 3564-3574.

Dobson, C.L., Warren, A.J., Pannell, R., Forster, A. and Rabbitts, T.H. (2000) Tumorigenesis in mice with a fusion of the leukaemia oncogene Mll and the bacterial lacZ gene. *Embo J*, **19**, 843-851.

Dolle, P., Izpisua-Belmonte, J.C., Brown, J., Tickle, C. and Duboule, D. (1993) Hox genes and the morphogenesis of the vertebrate limb. *Prog Clin Biol Res*, 11-20.

Domer, P.H., Fakharzadeh, S.S., Chen, C.S., Jockel, J., Johansen, L., Silverman, G.A., Kersey, J.H. and Korsmeyer, S.J. (1993) Acute mixed-lineage leukemia t(4;11)(q21;q23) generates an MLL-AF4 fusion product. *Proc Natl Acad Sci U S A*, **90**, 7884-7888.

Dordelmann, M., Reiter, A., Borkhardt, A., Ludwig, W.D., Gotz, N., Viehmann, S., Gadner, H., Riehm, H. and Schrappe, M. (1999) Prednisone response is the strongest predictor of treatment outcome in infant acute lymphoblastic leukemia. *Blood*, **94**, 1209-1217.

Duboule, D. (1995) Vertebrate Hox genes and proliferation: an alternative pathway to homeosis? *Curr Opin Genet Dev*, **5**, 525-528.

Duboule, D. and Morata, G. (1994) Colinearity and functional hierarchy among genes of the homeotic complexes. *Trends Genet*, **10**, 358-364.

Dunaief, J.L., Strober, B.E., Guha, S., Khavari, P.A., Alin, K., Luban, J., Begemann, M., Crabtree, G.R. and Goff, S.P. (1994) The retinoblastoma protein and BRG1 form a complex and cooperate to induce cell cycle arrest. *Cell*, **79**, 119-130.

Elenbaas, B., Spirio, L., Koerner, F., Fleming, M.D., Zimonjic, D.B., Donaher, J.L., Popescu, N.C., Hahn, W.C. and Weinberg, R.A. (2001) Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. *Genes Dev*, **15**, 50-65.

Ernst, P., Wang, J., Huang, M., Goodman, R.H. and Korsmeyer, S.J. (2001) MLL and CREB bind cooperatively to the nuclear coactivator CREB-binding protein. *Mol Cell Biol*, **21**, 2249-2258.

Fair, K., Anderson, M., Bulanova, E., Mi, H., Tropschug, M. and Diaz, M.O. (2001) Protein interactions of the MLL PHD fingers modulate MLL target gene regulation in human cells. *Mol Cell Biol*, **21**, 3589-3597.

Fanti, L., Giovinazzo, G., Berloco, M. and Pimpinelli, S. (1998) The heterochromatin protein 1 prevents telomere fusions in Drosophila. *Mol Cell*, **2**, 527-538.

Ferrandon, D., Jung, A.C., Criqui, M., Lemaitre, B., Uttenweiler-Joseph, S., Michaut, L., Reichhart, J. and Hoffmann, J.A. (1998) A drosomycin-GFP reporter transgene reveals a local immune response in Drosophila that is not dependent on the Toll pathway. *Embo J*, **17**, 1217-1227.

Fitzgerald, D.P. and Bender, W. (2001) Polycomb group repression reduces DNA accessibility. *Mol Cell Biol*, **21**, 6585-6597.

Francis, N.J. and Kingston, R.E. (2001) Mechanisms of transcriptional memory. *Nat Rev Mol Cell Biol*, **2**, 409-421.

Franke, A., DeCamillis, M., Zink, D., Cheng, N., Brock, H.W. and Paro, R. (1992) Polycomb and polyhomeotic are constituents of a multimeric protein complex in chromatin of Drosophila melanogaster. *Embo J*, **11**, 2941-2950.

Garcia-Bellido, A. (1975) Genetic control of wing disc development in Drosophila. *Ciba Found Symp*, **0**, 161-182.

Garcia-Cuellar, M.P., Zilles, O., Schreiner, S.A., Birke, M., Winkler, T.H. and Slany, R.K. (2001) The ENL moiety of the childhood leukemia-associated MLL-ENL oncoprotein recruits human Polycomb 3. *Oncogene*, **20**, 411-419.

Gasser, S.M., Amati, B.B., Cardenas, M.E. and Hofmann, J.F. (1989) Studies on scaffold attachment sites and their relation to genome function. *Int Rev Cytol*, **119**, 57-96.

Gateff, E. (1978) Malignant neoplasms of genetic origin in Drosophila melanogaster. *Science*, **200**, 1448-1459.

Gateff, E. (1994) Tumor-suppressor genes, hematopoietic malignancies and other hematopoietic disorders of Drosophila melanogaster. *Ann N Y Acad Sci*, **712**, 260-279.

Gateff, E. and Schneiderman, H.A. (1969) Neoplasms in mutant and cultured wild-tupe tissues of Drosophila. *Natl Cancer Inst Monogr*, **31**, 365-397.

Gatti, M. and Baker, B.S. (1989) Genes controlling essential cell-cycle functions in Drosophila melanogaster. *Genes Dev*, **3**, 438-453.

Gelmetti, V., Zhang, J., Fanelli, M., Minucci, S., Pelicci, P.G. and Lazar, M.A. (1998) Aberrant recruitment of the nuclear receptor corepressor-histone deacetylase complex by the acute myeloid leukemia fusion partner ETO. *Mol Cell Biol*, **18**, 7185-7191.

Gildea, J.J., Lopez, R. and Shearn, A. (2000) A screen for new trithorax group genes identified little imaginal discs, the Drosophila melanogaster homologue of human retinoblastoma binding protein 2. *Genetics*, **156**, 645-663.

Goto, H., Tomono, Y., Ajiro, K., Kosako, H., Fujita, M., Sakurai, M., Okawa, K., Iwamatsu, A., Okigaki, T., Takahashi, T. and Inagaki, M. (1999) Identification of a novel phosphorylation site on histone H3 coupled with mitotic chromosome condensation. *J Biol Chem*, **274**, 25543-25549.

Gould, A. (1997) Functions of mammalian Polycomb group and trithorax group related genes. *Curr Opin Genet Dev*, **7**, 488-494.

Gu, Y., Nakamura, T., Alder, H., Prasad, R., Canaani, O., Cimino, G., Croce, C.M. and Canaani, E. (1992) The t(4;11) chromosome translocation of human acute leukemias fuses the ALL-1 gene, related to Drosophila trithorax, to the AF-4 gene. *Cell*, **71**, 701-708.

Guo, C.Y., Mizzen, C., Wang, Y. and Larner, J.M. (2000) Histone H1 and H3 dephosphorylation are differentially regulated by radiation-induced signal transduction pathways. *Cancer Res*, **60**, 5667-5672.

Hanratty, W.P. and Dearolf, C.R. (1993) The Drosophila Tumorous-lethal hematopoietic oncogene is a dominant mutation in the hopscotch locus. *Mol Gen Genet*, **238**, 33-37.

Hanson, R.D., Hess, J.L., Yu, B.D., Ernst, P., van Lohuizen, M., Berns, A., van der Lugt, N.M., Shashikant, C.S., Ruddle, F.H., Seto, M. and Korsmeyer, S.J. (1999) Mammalian Trithorax and polycomb-group homologues are antagonistic regulators of homeotic development. *Proc Natl Acad Sci U S A*, **96**, 14372-14377.

Harbour, J.W. and Dean, D.C. (2000) Rb function in cell-cycle regulation and apoptosis. *Nat Cell Biol*, **2**, E65-67. taf/DynaPage.taf?file=/ncb/v62/n64/full/ncb0400_E0465.html taf/DynaPage.taf?file=/ncb/v0402/n0404/abs/ncb0400_E0465.html.

Harrison, C.J. (2001) Acute lymphoblastic leukaemia. *Best Pract Res Clin Haematol*, **14**, 593-607.

Harrison, D.A., Binari, R., Nahreini, T.S., Gilman, M. and Perrimon, N. (1995a) Activation of a Drosophila Janus kinase (JAK) causes hematopoietic neoplasia and developmental defects. *Embo J*, **14**, 2857-2865.

Harrison, D.A., McCoon, P.E., Binari, R., Gilman, M. and Perrimon, N. (1998) Drosophila unpaired encodes a secreted protein that activates the JAK signaling pathway. *Genes Dev*, **12**, 3252-3263.

Harrison, S.D., Solomon, N. and Rubin, G.M. (1995b) A genetic analysis of the 63E-64A genomic region of Drosophila melanogaster: identification of mutations in a replication factor C subunit. *Genetics*, **139**, 1701-1709.

Hashizume, R., Fukuda, M., Maeda, I., Nishikawa, H., Oyake, D., Yabuki, Y., Ogata, H. and Ohta, T. (2001) The RING heterodimer BRCA1-BARD1 is a ubiquitin ligase inactivated by a breast cancer-derived mutation. *J Biol Chem*, **276**, 14537-14540.

Hearst JE, Kauffman L, McClain WM. (1998) A simple mechanism for the avoidance of entanglement during chromosome replication. *Trends Genet.*, **14**, 244-7.

Heerema, N.A., Arthur, D.C., Sather, H., Albo, V., Feusner, J., Lange, B.J., Steinherz, P.G., Zeltzer, P., Hammond, D. and Reaman, G.H. (1994) Cytogenetic features of infants less than 12 months of age at diagnosis of acute lymphoblastic leukemia: impact of the 11q23 breakpoint on outcome: a report of the Childrens Cancer Group. *Blood*, **83**, 2274-2284.

Hemenway, C.S., de Erkenez, A.C. and Gould, G.C. (2001) The polycomb protein MPc3 interacts with AF9, an MLL fusion partner in t(9;11)(p22;q23) acute leukemias. *Oncogene*, **20**, 3798-3805.

Hendrich, B. and Bird, A. (1998) Identification and characterization of a family of mammalian methyl-CpG binding proteins. *Mol Cell Biol*, **18**, 6538-6547.

Herault, Y., Beckers, J., Gerard, M. and Duboule, D. (1999) Hox gene expression in limbs: colinearity by opposite regulatory controls. *Dev Biol*, **208**, 157-165.

Hidalgo, A., Urban, J. and Brand, A.H. (1995) Targeted ablation of glia disrupts axon tract formation in the Drosophila CNS. *Development*, **121**, 3703-3712.

Hodges, M., Tissot, C., Howe, K., Grimwade, D. and Freemont, P.S. (1998) Structure, organization, and dynamics of promyelocytic leukemia protein nuclear bodies. *Am J Hum Genet*, **63**, 297-304.

Hodgson, J.W., Argiropoulos, B. and Brock, H.W. (2001) Site-specific recognition of a 70-base-pair element containing d(GA)(n) repeats mediates bithoraxoid polycomb group response element-dependent silencing. *Mol Cell Biol*, **21**, 4528-4543.

Hoffmann, G. and Schobersberger, W. (1999) Anti-inflammatory and nitric oxide-inhibiting properties of granulocyte colony-stimulating factor. *Zhongguo Yao Li Xue Bao*, **20**, 673-681.

Huang, D.W., Fanti, L., Pak, D.T., Botchan, M.R., Pimpinelli, S. and Kellum, R. (1998) Distinct cytoplasmic and nuclear fractions of Drosophila heterochromatin protein 1: their phosphorylation levels and associations with origin recognition complex proteins. *J Cell Biol*, **142**, 307-318.

Humpherys, D., Eggan, K., Akutsu, H., Hochedlinger, K., Rideout, W.M., 3rd, Biniszkiewicz, D., Yanagimachi, R. and Jaenisch, R. (2001) Epigenetic instability in ES cells and cloned mice. *Science*, **293**, 95-97.

Huth, J.R., Bewley, C.A., Nissen, M.S., Evans, J.N., Reeves, R., Gronenborn, A.M. and Clore, G.M. (1997) The solution structure of an HMG-I(Y)-DNA complex defines a new architectural minor groove binding motif. *Nat Struct Biol*, **4**, 657-665.

Ingham, P.W. (1998) trithorax and the regulation of homeotic gene expression in Drosophila: a historical perspective. *Int J Dev Biol*, **42**, 423-429.

Ip, Y.T. and Levine, M. (1994) Molecular genetics of Drosophila immunity. *Curr Opin Genet Dev*, **4**, 672-677.

Isnard, P., Core, N., Naquet, P. and Djabali, M. (2000) Altered lymphoid development in mice deficient for the mAF4 proto- oncogene. *Blood*, **96**, 705-710.

Ito, K., Awano, W., Suzuki, K., Hiromi, Y. and Yamamoto, D. (1997) The Drosophila mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. *Development*, **124**, 761-771.

Jacobs, J.J., Keblusek, P., Robanus-Maandag, E., Kristel, P., Lingbeek, M., Nederlof, P.M., van Welsem, T., van de Vijver, M.J., Koh, E.Y., Daley, G.Q. and van Lohuizen, M. (2000) Senescence bypass screen identifies TBX2, which represses Cdkn2a (p19(ARF)) and is amplified in a subset of human breast cancers. *Nat Genet*, **26**, 291-299. taf/DynaPage.taf?file=/ng/journal/v226/n293/full/ng1100_1291.html taf/DynaPage.taf?file=/ng/journal/v1126/n1103/abs/ng1100_1291.html.

Jacobs, J.J., Kieboom, K., Marino, S., DePinho, R.A. and van Lohuizen, M. (1999a) The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. *Nature*, **397**, 164-168.

Jacobs, J.J., Scheijen, B., Voncken, J.W., Kieboom, K., Berns, A. and van Lohuizen, M. (1999b) Bmi-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc- induced apoptosis via INK4a/ARF. *Genes Dev*, **13**, 2678-2690.

Jenuwein, T. and Allis, C.D. (2001) Translating the histone code. *Science*, **293**, 1074-1080.

Jin, Y., Wang, Y., Walker, D.L., Dong, H., Conley, C., Johansen, J. and Johansen, K.M. (1999) JIL-1: a novel chromosomal tandem kinase implicated in transcriptional regulation in Drosophila. *Mol Cell*, **4**, 129-135.

Johansson, B., Moorman, A.V., Haas, O.A., Watmore, A.E., Cheung, K.L., Swanton, S. and Secker-Walker, L.M. (1998) Hematologic malignancies with t(4;11)(q21;q23)--a cytogenetic, morphologic, immunophenotypic and clinical study of 183 cases. European 11q23 Workshop participants. *Leukemia*, **12**, 779-787.

Johnstone, R.W., Gerber, M., Landewe, T., Tollefson, A., Wold, W.S. and Shilatifard, A. (2001) Functional analysis of the leukemia protein ELL: evidence for a role in the regulation of cell growth and survival. *Mol Cell Biol*, **21**, 1672-1681.

Jones, C.A., Ng, J., Peterson, A.J., Morgan, K., Simon, J. and Jones, R.S. (1998) The Drosophila esc and E(z) proteins are direct partners in polycomb group-mediated repression. *Mol Cell Biol*, **18**, 2825-2834.

Jones, R.S. and Gelbart, W.M. (1990) Genetic analysis of the enhancer of zeste locus and its role in gene regulation in Drosophila melanogaster. *Genetics*, **126**, 185-199.

Kal, A.J., Mahmoudi, T., Zak, N.B. and Verrijzer, C.P. (2000) The Drosophila brahma complex is an essential coactivator for the trithorax group protein zeste. *Genes Dev*, **14**, 1058-1071.

Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S.C., Heyman, R.A., Rose, D.W., Glass, C.K. and Rosenfeld, M.G. (1996) A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. *Cell*, **85**, 403-414.

Katsani, K.R., Arredondo, J.J., Kal, A.J. and Verrijzer, C.P. (2001) A homeotic mutation in the trithorax SET domain impedes histone binding. *Genes Dev*, **15**, 2197-2202.

Kawagoe, H., Kawagoe, R. and Sano, K. (2001) Targeted down-regulation of MLL-AF9 with antisense oligodeoxyribonucleotide reduces the expression of the HOXA7 and -A10 genes and induces apoptosis in a human leukemia cell line, THP-1. *Leukemia*, **15**, 1743-1749.

Khadake, J.R. and Rao, M.R. (1997) Preferential condensation of SAR-DNA by histone H1 and its SPKK containing octapeptide repeat motif. *FEBS Lett*, **400**, 193-196.

Klochendler-Yeivin, A. and Yaniv, M. (2001) Chromatin modifiers and tumor suppression. *Biochim Biophys Acta*, **1551**, M1-10.

Koerner, T.J., Hill, J.E., Myers, A.M. and Tzagoloff, A. (1991) High-expression vectors with multiple cloning sites for construction of trpE fusion genes: pATH vectors. *Methods Enzymol*, **194**, 477-490.

Kondo, T. and Duboule, D. (1999) Breaking colinearity in the mouse HoxD complex. *Cell*, **97**, 407-417.

Koshland, D. and Strunnikov, A. (1996) Mitotic chromosome condensation. *Annu Rev Cell Dev Biol*, **12**, 305-333.

Kostic, D. and Capecchi, M.R. (1994) Targeted disruptions of the murine Hoxa-4 and Hoxa-6 genes result in homeotic transformations of components of the vertebral column. *Mech Dev*, **46**, 231-247.

Krause, S.A., Loupart, M.L., Vass, S., Schoenfelder, S., Harrison, S. and Heck, M.M. (2001) Loss of cell cycle checkpoint control in Drosophila Rfc4 mutants. *Mol Cell Biol*, **21**, 5156-5168.

Krebs, J.E., Kuo, M.H., Allis, C.D. and Peterson, C.L. (1999) Cell cycle-regulated histone acetylation required for expression of the yeast HO gene. *Genes Dev*, **13**, 1412-1421.

Krimpenfort, P., Quon, K.C., Mooi, W.J., Loonstra, A. and Berns, A. (2001) Loss of p16Ink4a confers susceptibility to metastatic melanoma in mice. *Nature*, **413**, 83-86.

Kuo, M.H., Brownell, J.E., Sobel, R.E., Ranalli, T.A., Cook, R.G., Edmondson, D.G., Roth, S.Y. and Allis, C.D. (1996) Transcription-linked acetylation by Gcn5p of histones H3 and H4 at specific lysines. *Nature*, **383**, 269-272.

Kuzin, B., Tillib, S., Sedkov, Y., Mizrokhi, L. and Mazo, A. (1994) The Drosophila trithorax gene encodes a chromosomal protein and directly regulates the region-specific homeotic gene fork head. *Genes Dev*, **8**, 2478-2490.

Lachner, M., O'Carroll, D., Rea, S., Mechtler, K. and Jenuwein, T. (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature*, **410**, 116-120.

LaJeunesse, D. and Shearn, A. (1996) E(z): a polycomb group gene or a trithorax group gene? *Development*, **122**, 2189-2197.

Lavau, C., Luo, R.T., Du, C. and Thirman, M.J. (2000) Retrovirus-mediated gene transfer of MLL-ELL transforms primary myeloid progenitors and causes acute myeloid leukemias in mice. *Proc Natl Acad Sci U S A*, **97**, 10984-10989.

Lawrence, P.A. and Morata, G. (1994) Homeobox genes: their function in Drosophila segmentation and pattern formation. *Cell*, **78**, 181-189.

Lawson, K.A., Meneses, J.J. and Pedersen, R.A. (1991) Clonal analysis of epiblast fate during germ layer formation in the mouse embryo. *Development*, **113**, 891-911.

Lebestky, T., Chang, T., Hartenstein, V. and Banerjee, U. (2000) Specification of Drosophila hematopoietic lineage by conserved transcription factors. *Science*, **288**, 146-149.

Lee, S.J., Choi, J.Y., Sung, Y.M., Park, H., Rhim, H. and Kang, S. (2001) E3 ligase activity of RING finger proteins that interact with Hip-2, a human ubiquitin-conjugating enzyme. *FEBS Lett*, **503**, 61-64.

Lessard, J., Schumacher, A., Thorsteinsdottir, U., van Lohuizen, M., Magnuson, T. and Sauvageau, G. (1999) Functional antagonism of the Polycomb-Group genes eed and Bmi1 in hemopoietic cell proliferation. *Genes Dev*, **13**, 2691-2703.

Lin, Y.M., Ono, K., Satoh, S., Ishiguro, H., Fujita, M., Miwa, N., Tanaka, T., Tsunoda, T., Yang, K.C., Nakamura, Y. and Furukawa, Y. (2001) Identification of AF17 as a downstream gene of the beta-catenin/T-cell factor pathway and its involvement in colorectal carcinogenesis. *Cancer Res*, **61**, 6345-6349.

Linder, B., Gerlach, N. and Jackle, H. (2001) The Drosophila homolog of the human AF10 is an HP1-interacting suppressor of position effect variegation. *EMBO Rep*, **2**, 211-216.

Lipford, J.R. and Bell, S.P. (2001) Nucleosomes positioned by ORC facilitate the initiation of DNA replication. *Mol Cell*, **7**, 21-30.

Litt, M.D., Simpson, M., Gaszner, M., Allis, C.D. and Felsenfeld, G. (2001a) Correlation between histone lysine methylation and developmental changes at the chicken beta-globin locus. *Science*, **293**, 2453-2455.

Litt, M.D., Simpson, M., Recillas-Targa, F., Prioleau, M.N. and Felsenfeld, G. (2001b) Transitions in histone acetylation reveal boundaries of three separately regulated neighboring loci. *Embo J*, **20**, 2224-2235.

Loupart, M.L., Krause, S.A. and Heck, M.S. (2000) Aberrant replication timing induces defective chromosome condensation in Drosophila ORC2 mutants. *Curr Biol*, **10**, 1547-1556.

Luo, J., Su, F., Chen, D., Shiloh, A. and Gu, W. (2000) Deacetylation of p53 modulates its effect on cell growth and apoptosis. *Nature*, **408**, 377-381.

Luo, R.T., Lavau, C., Du, C., Simone, F., Polak, P.E., Kawamata, S. and Thirman, M.J. (2001) The elongation domain of ELL is dispensable but its ELL-associated factor 1 interaction domain is essential for MLL-ELL-induced leukemogenesis. *Mol Cell Biol*, **21**, 5678-5687.

Lupo, R., Breiling, A., Bianchi, M.E. and Orlando, V. (2001) Drosophila chromosome condensation proteins Topoisomerase II and Barren colocalize with Polycomb and maintain Fab-7 PRE silencing. *Mol Cell*, **7**, 127-136.

Ma, C. and Staudt, L.M. (1996) LAF-4 encodes a lymphoid nuclear protein with transactivation potential that is homologous to AF-4, the gene fused to MLL in t(4;11) leukemias. *Blood*, **87**, 734-745.

Ma, Q., Alder, H., Nelson, K.K., Chatterjee, D., Gu, Y., Nakamura, T., Canaani, E., Croce, C.M., Siracusa, L.D. and Buchberg, A.M. (1993) Analysis of the murine All-1 gene reveals conserved domains with human ALL-1 and identifies a motif shared with DNA methyltransferases. *Proc Natl Acad Sci U S A*, **90**, 6350-6354.

Mahadevan, L.C., Willis, A.C. and Barratt, M.J. (1991) Rapid histone H3 phosphorylation in response to growth factors, phorbol esters, okadaic acid, and protein synthesis inhibitors. *Cell*, **65**, 775-783.

Mann, R.S. and Morata, G. (2000) The developmental and molecular biology of genes that subdivide the body of Drosophila. *Annu Rev Cell Dev Biol*, **16**, 243-271.

Mathey-Prevot, B. and Perrimon, N. (1998) Mammalian and Drosophila blood: JAK of all trades? *Cell*, **92**, 697-700.

McCall, K. and Bender, W. (1996) Probes of chromatin accessibility in the Drosophila bithorax complex respond differently to Polycomb-mediated repression. *Embo J*, **15**, 569-580. abs.html.

Meister, M., Lemaitre, B. and Hoffmann, J.A. (1997) Antimicrobial peptide defense in Drosophila. *Bioessays*, **19**, 1019-1026.

Morata, G. and Lawrence, P.A. (1975) Control of compartment development by the engrailed gene in Drosophila. *Nature*, **255**, 614-617.

Morrissey, J.J., Raney, S. and Cleary, M.L. (1997) The FEL (AF-4) protein donates transcriptional activation sequences to Hrx-Fel fusion proteins in leukemias containing T(4;11)(Q21;Q23) chromosomal translocations. *Leuk Res*, **21**, 911-917.

Muchardt, C., Bourachot, B., Reyes, J.C. and Yaniv, M. (1998) ras transformation is associated with decreased expression of the brm/SNF2alpha ATPase from the mammalian SWI-SNF complex. *Embo J*, **17**, 223-231.

Muller, J., Gaunt, S. and Lawrence, P.A. (1995) Function of the Polycomb protein is conserved in mice and flies. *Development*, **121**, 2847-2852.

Munshi, N., Agalioti, T., Lomvardas, S., Merika, M., Chen, G. and Thanos, D. (2001) Coordination of a transcriptional switch by HMGI(Y) acetylation. *Science*, **293**, 1133-1136.

Muyrers JP, Zhang Y, Stewart AF. (2001) Techniques: Recombinogenic engineering--new options for cloning and manipulating DNA. *Trends Biochem Sci.*, **26**, 325-31.

Nakamura, T., Alder, H., Gu, Y., Prasad, R., Canaani, O., Kamada, N., Gale, R.P., Lange, B., Crist, W.M., Nowell, P.C. and et al. (1993) Genes on chromosomes 4, 9, and 19 involved in 11q23 abnormalities in acute leukemia share sequence homology and/or common motifs. *Proc Natl Acad Sci U S A*, **90**, 4631-4635.

Nakayama, J., Rice, J.C., Strahl, B.D., Allis, C.D. and Grewal, S.I. (2001) Role of histone H3 lysine 9 methylation in epigenetic control of heterochromatin assembly. *Science*, **292**, 110-113.

Ng, J., Hart, C.M., Morgan, K. and Simon, J.A. (2000) A Drosophila ESC-E(Z) protein complex is distinct from other polycomb group complexes and contains covalently modified ESC. *Mol Cell Biol*, **20**, 3069-3078.

Nielsen, S.J., Schneider, R., Bauer, U.M., Bannister, A.J., Morrison, A., O'Carroll, D., Firestein, R., Cleary, M., Jenuwein, T., Herrera, R.E. and Kouzarides, T. (2001) Rb targets histone H3 methylation and HP1 to promoters. *Nature*, **412**, 561-565.

Niitsu, N., Hayashi, Y. and Honma, Y. (2001) Downregulation of MLL-CBP fusion gene expression is associated with differentiation of SN-1 cells with t(11;16)(q23;p13). *Oncogene*, **20**, 375-384.

Nissen, M.S., Langan, T.A. and Reeves, R. (1991) Phosphorylation by cdc2 kinase modulates DNA binding activity of high mobility group I nonhistone chromatin protein. *J Biol Chem*, **266**, 19945-19952.

Noma, K., Allis, C.D. and Grewal, S.I. (2001) Transitions in distinct histone H3 methylation patterns at the heterochromatin domain boundaries. *Science*, **293**, 1150-1155.

Onishi, A., Iwamoto, M., Akita, T., Mikawa, S., Takeda, K., Awata, T., Hanada, H. and Perry, A.C. (2000) Pig cloning by microinjection of fetal fibroblast nuclei. *Science*, **289**, 1188-1190.

Orlando, V., Jane, E.P., Chinwalla, V., Harte, P.J. and Paro, R. (1998) Binding of trithorax and Polycomb proteins to the bithorax complex: dynamic changes during early Drosophila embryogenesis. *Embo J*, **17**, 5141-5150.

Orlando, V. and Paro, R. (1993) Mapping Polycomb-repressed domains in the bithorax complex using in vivo formaldehyde cross-linked chromatin. *Cell*, **75**, 1187-1198.

Owen, D.J., Ornaghi, P., Yang, J.C., Lowe, N., Evans, P.R., Ballario, P., Neuhaus, D., Filetici, P. and Travers, A.A. (2000) The structural basis for the recognition of acetylated histone H4 by the bromodomain of histone acetyltransferase gcn5p. *Embo J*, **19**, 6141-6149.

Pak, D.T., Pflumm, M., Chesnokov, I., Huang, D.W., Kellum, R., Marr, J., Romanowski, P. and Botchan, M.R. (1997) Association of the origin recognition complex with heterochromatin and HP1 in higher eukaryotes. *Cell*, **91**, 311-323.

Papoulas, O., Beek, S.J., Moseley, S.L., McCallum, C.M., Sarte, M., Shearn, A. and Tamkun, J.W. (1998) The Drosophila trithorax group proteins BRM, ASH1 and ASH2 are subunits of distinct protein complexes. *Development*, **125**, 3955-3966.

Paro, R. (1990) Imprinting a determined state into the chromatin of Drosophila. *Trends Genet*, **6**, 416-421.

Paro, R. and Hogness, D.S. (1991) The Polycomb protein shares a homologous domain with a heterochromatin-associated protein of Drosophila. *Proc Natl Acad Sci U S A*, **88**, 263-267.

Partanen, J., Schwartz, L. and Rossant, J. (1998) Opposite phenotypes of hypomorphic and Y766 phosphorylation site mutations reveal a function for Fgfr1 in anteroposterior patterning of mouse embryos. *Genes Dev*, **12**, 2332-2344.

Peters, A.H., O'Carroll, D., Scherthan, H., Mechtler, K., Sauer, S., Schofer, C., Weipoltshammer, K., Pagani, M., Lachner, M., Kohlmaier, A., Opravil, S., Doyle, M., Sibilia, M. and Jenuwein, T. (2001) Loss of the Suv39h histone methyltransferases impairs mammalian heterochromatin and genome stability. *Cell*, **107**, 323-337.

Petruk, S., Sedkov, Y., Smith, S., Tillib, S., Kraevski, V., Nakamura, T., Canaani, E., Croce, C.M. and Mazo, A. (2001) Trithorax and dCBP acting in a complex to maintain expression of a homeotic gene. *Science*, **294**, 1331-1334.

Pflumm, M.F. and Botchan, M.R. (2001) Orc mutants arrest in metaphase with abnormally condensed chromosomes. *Development*, **128**, 1697-1707.

Pirrotta, V. (1998) Polycombing the genome: PcG, trxG, and chromatin silencing. *Cell*, **93**, 333-336.

Platero, J.S., Hartnett, T. and Eissenberg, J.C. (1995) Functional analysis of the chromo domain of HP1. *Embo J*, **14**, 3977-3986.

Polejaeva, I.A., Chen, S.H., Vaught, T.D., Page, R.L., Mullins, J., Ball, S., Dai, Y., Boone, J., Walker, S., Ayares, D.L., Colman, A. and Campbell, K.H. (2000) Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature*, **407**, 86-90.

Poux, S., Kostic, C. and Pirrotta, V. (1996) Hunchback-independent silencing of late Ubx enhancers by a Polycomb Group Response Element. *Embo J*, **15**, 4713-4722.

Poux, S., McCabe, D. and Pirrotta, V. (2001a) Recruitment of components of Polycomb Group chromatin complexes in Drosophila. *Development*, **128**, 75-85.

Poux, S., Melfi, R. and Pirrotta, V. (2001b) Establishment of Polycomb silencing requires a transient interaction between PC and ESC. *Genes Dev*, **15**, 2509-2514.

Prasad, R., Yano, T., Sorio, C., Nakamura, T., Rallapalli, R., Gu, Y., Leshkowitz, D., Croce, C.M. and Canaani, E. (1995) Domains with transcriptional regulatory activity within the ALL1 and AF4 proteins involved in acute leukemia. *Proc Natl Acad Sci U S A*, **92**, 12160-12164.

Pui, C.H. (2000) Acute lymphoblastic leukemia in children. *Curr Opin Oncol*, **12**, 3-12.

Pui, C.H., Behm, F.G., Downing, J.R., Hancock, M.L., Shurtleff, S.A., Ribeiro, R.C., Head, D.R., Mahmoud, H.H., Sandlund, J.T., Furman, W.L. and et al. (1994) 11q23/MLL rearrangement confers a poor prognosis in infants with acute lymphoblastic leukemia. *J Clin Oncol*, **12**, 909-915.

Pui, C.H., Kane, J.R. and Crist, W.M. (1995) Biology and treatment of infant leukemias. *Leukemia*, **9**, 762-769.

Rabbitts, T.H. (1994) Chromosomal translocations in human cancer. *Nature*, **372**, 143-149.

Rabbitts, T.H., Appert, A., Chung, G., Collins, E.C., Drynan, L., Forster, A., Lobato, M.N., McCormack, M.P., Pannell, R., Spandidos, A., Stocks, M.R., Tanaka, T. and Tse, E. (2001) Mouse models of human chromosomal translocations and approaches to cancer therapy. *Blood Cells Mol Dis*, **27**, 249-259.

Rastelli, L., Chan, C.S. and Pirrotta, V. (1993) Related chromosome binding sites for zeste, suppressors of zeste and Polycomb group proteins in Drosophila and their dependence on Enhancer of zeste function. *Embo J*, **12**, 1513-1522.

Rea S, Eisenhaber F, O'Carroll D, Strahl BD, Sun ZW, Schmid M, Opravil S, Mechtler K, Ponting CP, Allis CD, Jenuwein T. (2000) Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature*, **406(6796)**, 593-9.

Reeves, R., Langan, T.A. and Nissen, M.S. (1991) Phosphorylation of the DNA-binding domain of nonhistone high-mobility group I protein by cdc2 kinase: reduction of binding affinity. *Proc Natl Acad Sci U S A*, **88**, 1671-1675.

Reeves, R. and Nissen, M.S. (1990) The A.T-DNA-binding domain of mammalian high mobility group I chromosomal proteins. A novel peptide motif for recognizing DNA structure. *J Biol Chem*, **265**, 8573-8582.

Reyes, J.C., Muchardt, C. and Yaniv, M. (1997) Components of the human SWI/SNF complex are enriched in active chromatin and are associated with the nuclear matrix. *J Cell Biol*, **137**, 263-274.

Rice, J.C. and Futscher, B.W. (2000) Transcriptional repression of BRCA1 by aberrant cytosine methylation, histone hypoacetylation and chromatin condensation of the BRCA1 promoter. *Nucleic Acids Res*, **28**, 3233-3239.

Rideout, W.M., 3rd, Eggan, K. and Jaenisch, R. (2001) Nuclear cloning and epigenetic reprogramming of the genome. *Science*, **293**, 1093-1098.

Riede, I. (1996) Three mutant genes cooperatively induce brain tumor formation in Drosophila malignant brain tumor. *Cancer Genet Cytogenet*, **90**, 135-141.

Rijli, F.M., Mark, M., Lakkaraju, S., Dierich, A., Dolle, P. and Chambon, P. (1993) A homeotic transformation is generated in the rostral branchial region of the head by disruption of Hoxa-2, which acts as a selector gene. *Cell*, **75**, 1333-1349.

Rogakou, E.P., Boon, C., Redon, C. and Bonner, W.M. (1999) Megabase chromatin domains involved in DNA double-strand breaks in vivo. *J Cell Biol*, **146**, 905-916.

Romanov, S.R., Kozakiewicz, B.K., Holst, C.R., Stampfer, M.R., Haupt, L.M. and Tlsty, T.D. (2001) Normal human mammary epithelial cells spontaneously escape senescence and acquire genomic changes. *Nature*, **409**, 633-637.

Rowley, J.D. (1998) The critical role of chromosome translocations in human leukemias. *Annu Rev Genet*, **32**, 495-519.

Rozenblatt-Rosen, O., Rozovskaia, T., Burakov, D., Sedkov, Y., Tillib, S., Blechman, J., Nakamura, T., Croce, C.M., Mazo, A. and Canaani, E. (1998) The C-terminal SET domains of ALL-1 and TRITHORAX interact with the INI1 and SNR1 proteins, components of the SWI/SNF complex. *Proc Natl Acad Sci U S A*, **95**, 4152-4157.

Rozovskaia, T., Rozenblatt-Rosen, O., Sedkov, Y., Burakov, D., Yano, T., Nakamura, T., Petruck, S., Ben-Simchon, L., Croce, C.M., Mazo, A. and Canaani, E. (2000) Self-association of the SET domains of human ALL-1 and of Drosophila TRITHORAX and ASH1 proteins. *Oncogene*, **19**, 351-357.

Rozovskaia, T., Tillib, S., Smith, S., Sedkov, Y., Rozenblatt-Rosen, O., Petruk, S., Yano, T., Nakamura, T., Ben-Simchon, L., Gildea, J., Croce, C.M., Shearn, A., Canaani, E. and Mazo, A. (1999) Trithorax and ASH1 interact directly and associate with the trithorax group-responsive bxd region of the Ultrabithorax promoter. *Mol Cell Biol*, **19**, 6441-6447.

Rubnitz, J.E., Morrissey, J., Savage, P.A. and Cleary, M.L. (1994) ENL, the gene fused with HRX in t(11;19) leukemias, encodes a nuclear protein with transcriptional activation potential in lymphoid and myeloid cells. *Blood*, **84**, 1747-1752.

Ruhf, M.L., Braun, A., Papoulas, O., Tamkun, J.W., Randsholt, N. and Meister, M. (2001) The domino gene of Drosophila encodes novel members of the SWI2/SNF2 family of DNA-dependent ATPases, which contribute to the silencing of homeotic genes. *Development*, **128**, 1429-1441.

Rundlett, S.E., Carmen, A.A., Suka, N., Turner, B.M. and Grunstein, M. (1998) Transcriptional repression by UME6 involves deacetylation of lysine 5 of histone H4 by RPD3. *Nature*, **392**, 831-835.

Saha, V., Young, B.D. and Freemont, P.S. (1998) Translocations, fusion genes, and acute leukemia. *J Cell Biochem Suppl*, **31**, 264-276.

Satijn, D.P., Gunster, M.J., van der Vlag, J., Hamer, K.M., Schul, W., Alkema, M.J., Saurin, A.J., Freemont, P.S., van Driel, R. and Otte, A.P. (1997) RING1 is associated with the polycomb group protein complex and acts as a transcriptional repressor. *Mol Cell Biol*, **17**, 4105-4113.

Satijn, D.P. and Otte, A.P. (1999) RING1 interacts with multiple Polycomb-group proteins and displays tumorigenic activity. *Mol Cell Biol*, **19**, 57-68.

Saurin, A.J., Shao, Z., Erdjument-Bromage, H., Tempst, P. and Kingston, R.E. (2001) A Drosophila Polycomb group complex includes Zeste and dTAFII proteins. *Nature*, **412**, 655-660.

Sawyer, M., Antoniou, G., Toogood, I. and Rice, M. (1997) Childhood cancer: a two-year prospective study of the psychological adjustment of children and parents. *J Am Acad Child Adolesc Psychiatry*, **36**, 1736-1743.

Schreiner, S., Birke, M., Garcia-Cuellar, M.P., Zilles, O., Greil, J. and Slany, R.K. (2001) MLL-ENL causes a reversible and myc-dependent block of myelomonocytic cell differentiation. *Cancer Res*, **61**, 6480-6486.

Sekinger, E.A. and Gross, D.S. (2001) Silenced chromatin is permissive to activator binding and PIC recruitment. *Cell*, **105**, 403-414.

Seo, S.B., McNamara, P., Heo, S., Turner, A., Lane, W.S. and Chakravarti, D. (2001) Regulation of histone acetylation and transcription by INHAT, a human cellular complex containing the set oncoprotein. *Cell*, **104**, 119-130.

Sewalt, R.G., van der Vlag, J., Gunster, M.J., Hamer, K.M., den Blaauwen, J.L., Satijn, D.P., Hendrix, T., van Driel, R. and Otte, A.P. (1998) Characterization of interactions between the mammalian polycomb-group proteins Enx1/EZH2 and EED suggests the existence of different mammalian polycomb-group protein complexes. *Mol Cell Biol*, **18**, 3586-3595.

Shao, Z., Raible, F., Mollaaghababa, R., Guyon, J.R., Wu, C.T., Bender, W. and Kingston, R.E. (1999) Stabilization of chromatin structure by PRC1, a Polycomb complex. *Cell*, **98**, 37-46.

Shareef, M.M., King, C., Damaj, M., Badagu, R., Huang, D.W. and Kellum, R. (2001) Drosophila heterochromatin protein 1 (HP1)/origin recognition complex (ORC) protein is associated with HP1 and ORC and functions in heterochromatin-induced silencing. *Mol Biol Cell*, **12**, 1671-1685.

Sharp, E.J., Abramova, N.A., Park, W.J. and Adler, P.N. (1997) The conserved HR domain of the Drosophila suppressor 2 of zeste [Su(z)2] and murine bmi-1 proteins constitutes a locus-specific chromosome binding domain. *Chromosoma*, **106**, 70-80.

Sharpless, N.E., Bardeesy, N., Lee, K.H., Carrasco, D., Castrillon, D.H., Aguirre, A.J., Wu, E.A., Horner, J.W. and DePinho, R.A. (2001) Loss of p16Ink4a with retention of p19Arf predisposes mice to tumorigenesis. *Nature*, **413**, 86-91.

Sherr, C.J. (2001) Parsing Ink4a/Arf: "pure" p16-null mice. *Cell*, **106**, 531-534.

Shilatifard, A., Haque, D., Conaway, R.C. and Conaway, J.W. (1997) Structure and function of RNA polymerase II elongation factor ELL. Identification of two overlapping ELL functional domains that govern its interaction with polymerase and the ternary elongation complex. *J Biol Chem*, **272**, 22355-22363.

Shilatifard, A., Lane, W.S., Jackson, K.W., Conaway, R.C. and Conaway, J.W. (1996) An RNA polymerase II elongation factor encoded by the human ELL gene. *Science*, **271**, 1873-1876.

Shumacher, A., Faust, C. and Magnuson, T. (1996) Positional cloning of a global regulator of anterior-posterior patterning in mice. *Nature*, **383**, 250-253.

Simeone, A., Acampora, D., Arcioni, L., Andrews, P.W., Boncinelli, E. and Mavilio, F. (1990) Sequential activation of HOX2 homeobox genes by retinoic acid in human embryonal carcinoma cells. *Nature*, **346**, 763-766.

Simon, J. (1995) Locking in stable states of gene expression: transcriptional control during Drosophila development. *Curr Opin Cell Biol*, **7**, 376-385.

Simon, J., Bornemann, D., Lunde, K. and Schwartz, C. (1995) The extra sex combs product contains WD40 repeats and its time of action implies a role distinct from other Polycomb group products. *Mech Dev*, **53**, 197-208.

Simone, F., Polak, P.E., Kaberlein, J.J., Luo, R.T., Levitan, D.A. and Thirman, M.J. (2001) EAF1, a novel ELL-associated factor that is delocalized by expression of the MLL-ELL fusion protein. *Blood*, **98**, 201-209.

Slany, R.K., Lavau, C. and Cleary, M.L. (1998) The oncogenic capacity of HRX-ENL requires the transcriptional transactivation activity of ENL and the DNA binding motifs of HRX. *Mol Cell Biol*, **18**, 122-129.

Sobel, R.E., Cook, R.G., Perry, C.A., Annunziato, A.T. and Allis, C.D. (1995) Conservation of deposition-related acetylation sites in newly synthesized histones H3 and H4. *Proc Natl Acad Sci U S A*, **92**, 1237-1241.

Stassen, M.J., Bailey, D., Nelson, S., Chinwalla, V. and Harte, P.J. (1995) The Drosophila trithorax proteins contain a novel variant of the nuclear receptor type DNA binding domain and an ancient conserved motif found in other chromosomal proteins. *Mech Dev*, **52**, 209-223.

Strahl, B.D. and Allis, C.D. (2000) The language of covalent histone modifications. *Nature*, **403**, 41-45.

Strahl, B.D., Ohba, R., Cook, R.G. and Allis, C.D. (1999) Methylation of histone H3 at lysine 4 is highly conserved and correlates with transcriptionally active nuclei in Tetrahymena. *Proc Natl Acad Sci U S A*, **96**, 14967-14972.

Struhl, G. and Brower, D. (1982) Early role of the esc+ gene product in the determination of segments in Drosophila. *Cell*, **31**, 285-292.

Strutt, H., Cavalli, G. and Paro, R. (1997) Co-localization of Polycomb protein and GAGA factor on regulatory elements responsible for the maintenance of homeotic gene expression. *Embo J*, **16**, 3621-3632.

Strutt, H. and Paro, R. (1997) The polycomb group protein complex of Drosophila melanogaster has different compositions at different target genes. *Mol Cell Biol*, **17**, 6773-6783.

Stunnenberg, H.G., Garcia-Jimenez, C. and Betz, J.L. (1999) Leukemia: the sophisticated subversion of hematopoiesis by nuclear receptor oncoproteins. *Biochim Biophys Acta*, **1423**, F15-33.

Subramanian, V., Meyer, B.I. and Gruss, P. (1995) Disruption of the murine homeobox gene Cdx1 affects axial skeletal identities by altering the mesodermal expression domains of Hox genes. *Cell*, **83**, 641-653.

Sundin O, Varshavsky A. (1980)Terminal stages of SV40 DNA replication proceed via multiply intertwined catenated dimers. *Cell*, **21**, 103-14.

Taddei, A., Roche, D., Sibarita, J.B., Turner, B.M. and Almouzni, G. (1999) Duplication and maintenance of heterochromatin domains. *J Cell Biol*, **147**, 1153-1166.

Taki, T., Kano, H., Taniwaki, M., Sako, M., Yanagisawa, M. and Hayashi, Y. (1999) AF5q31, a newly identified AF4-related gene, is fused to MLL in infant acute lymphoblastic leukemia with ins(5;11)(q31;q13q23). *Proc Natl Acad Sci U S A*, **96**, 14535-14540.

Tam, P.P. and Beddington, R.S. (1987) The formation of mesodermal tissues in the mouse embryo during gastrulation and early organogenesis. *Development*, **99**, 109-126.

Tamaru, H. and Selker, E.U. (2001) A histone H3 methyltransferase controls DNA methylation in Neurospora crassa. *Nature*, **414**, 277-283.

Tamkun, J.W., Deuring, R., Scott, M.P., Kissinger, M., Pattatucci, A.M., Kaufman, T.C. and Kennison, J.A. (1992) brahma: a regulator of Drosophila homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2. *Cell*, **68**, 561-572.

Tang, A.H., Neufeld, T.P., Rubin, G.M. and Muller, H.A. (2001) Transcriptional regulation of cytoskeletal functions and segmentation by a novel maternal pair-rule gene, lilliputian. *Development*, **128**, 801-813.

Tie, F., Furuyama, T. and Harte, P.J. (1998) The Drosophila Polycomb Group proteins ESC and E(Z) bind directly to each other and co-localize at multiple chromosomal sites. *Development*, **125**, 3483-3496.

Tie, F., Furuyama, T., Prasad-Sinha, J., Jane, E. and Harte, P.J. (2001) The Drosophila Polycomb Group proteins ESC and E(Z) are present in a complex containing the histone-binding protein p55 and the histone deacetylase RPD3. *Development*, **128**, 275-286.

Tkachuk, D.C., Kohler, S. and Cleary, M.L. (1992) Involvement of a homolog of Drosophila trithorax by 11q23 chromosomal translocations in acute leukemias. *Cell*, **71**, 691-700.

Torok, I., Hartenstein, K., Kalmes, A., Schmitt, R., Strand, D. and Mechler, B.M. (1993) The l(2)gl homologue of Drosophila pseudoobscura suppresses tumorigenicity in transgenic Drosophila melanogaster. *Oncogene*, **8**, 1537-1549.

Torok T, Harvie PD, Buratovich M, Bryant PJ. (1997)The product of proliferation disrupter is concentrated at centromeres and required for mitotic chromosome condensation and cell proliferation in Drosophila. *Genes Dev.*, **11**, 213-25.

Tripoulas, N., LaJeunesse, D., Gildea, J. and Shearn, A. (1996) The Drosophila ash1 gene product, which is localized at specific sites on polytene chromosomes, contains a SET domain and a PHD finger. *Genetics*, **143**, 913-928.

Tripoulas, N.A., Hersperger, E., La Jeunesse, D. and Shearn, A. (1994) Molecular genetic analysis of the Drosophila melanogaster gene absent, small or homeotic discs1 (ash1). *Genetics*, **137**, 1027-1038.

Tschiersch, B., Hofmann, A., Krauss, V., Dorn, R., Korge, G. and Reuter, G. (1994) The protein encoded by the Drosophila position-effect variegation suppressor gene Su(var)3-9 combines domains of antagonistic regulators of homeotic gene complexes. *Embo J*, **13**, 3822-3831.

Tsukiyama, T. and Wu, C. (1995) Purification and properties of an ATP-dependent nucleosome remodeling factor. *Cell*, **83**, 1011-1020.

Turner, B.M. (2000) Histone acetylation and an epigenetic code. *Bioessays*, **22**, 836-845.

Turner, B.M., Birley, A.J. and Lavender, J. (1992) Histone H4 isoforms acetylated at specific lysine residues define individual chromosomes and chromatin domains in Drosophila polytene nuclei. *Cell*, **69**, 375-384.

van der Lugt, N.M., Alkema, M., Berns, A. and Deschamps, J. (1996) The Polycomb-group homolog Bmi-1 is a regulator of murine Hox gene expression. *Mech Dev*, **58**, 153-164.

van Kemenade, F.J., Raaphorst, F.M., Blokzijl, T., Fieret, E., Hamer, K.M., Satijn, D.P., Otte, A.P. and Meijer, C.J. (2001) Coexpression of BMI-1 and EZH2 polycomb-group proteins is associated with cycling cells and degree of malignancy in B-cell non-Hodgkin lymphoma. *Blood*, **97**, 3896-3901.

van Lohuizen, M. (1999) The trithorax-group and polycomb-group chromatin modifiers: implications for disease. *Curr Opin Genet Dev*, **9**, 355-361.

van Lohuizen, M., Frasch, M., Wientjens, E. and Berns, A. (1991a) Sequence similarity between the mammalian bmi-1 proto-oncogene and the Drosophila regulatory genes Psc and Su(z)2. *Nature*, **353**, 353-355.

van Lohuizen, M., Tijms, M., Voncken, J.W., Schumacher, A., Magnuson, T. and Wientjens, E. (1998) Interaction of mouse polycomb-group (Pc-G) proteins Enx1 and Enx2 with Eed: indication for separate Pc-G complexes. *Mol Cell Biol*, **18**, 3572-3579.

van Lohuizen, M., Verbeek, S., Scheijen, B., Wientjens, E., van der Gulden, H. and Berns, A. (1991b) Identification of cooperating oncogenes in E mu-myc transgenic mice by provirus tagging. *Cell*, **65**, 737-752.

Vassetzky, Y., Hair, A. and Mechali, M. (2000) Rearrangement of chromatin domains during development in Xenopus. *Genes Dev*, **14**, 1541-1552.

Versteege, I., Sevenet, N., Lange, J., Rousseau-Merck, M.F., Ambros, P., Handgretinger, R., Aurias, A. and Delattre, O. (1998) Truncating mutations of hSNF5/INI1 in aggressive paediatric cancer. *Nature*, **394**, 203-206.

Vignali, M., Hassan, A.H., Neely, K.E. and Workman, J.L. (2000) ATP-dependent chromatin-remodeling complexes. *Mol Cell Biol*, **20**, 1899-1910.

von Bergh, A., Gargallo, P., De Prijck, B., Vranckx, H., Marschalek, R., Larripa, I., Kluin, P., Schuuring, E. and Hagemeijer, A. (2001) Cryptic t(4;11) encoding MLL-AF4 due to insertion of 5' MLL sequences in chromosome 4. *Leukemia*, **15**, 595-600.

Vonlanthen, S., Heighway, J., Altermatt, H.J., Gugger, M., Kappeler, A., Borner, M.M., van Lohuizen, M. and Betticher, D.C. (2001) The bmi-1 oncoprotein is differentially expressed in non-small cell lung cancer and correlates with INK4A-ARF locus expression. *Br J Cancer*, **84**, 1372-1376.

Wang, H., Huang, Z.Q., Xia, L., Feng, Q., Erdjument-Bromage, H., Strahl, B.D., Briggs, S.D., Allis, C.D., Wong, J., Tempst, P. and Zhang, Y. (2001) Methylation of histone H4 at arginine 3 facilitating transcriptional activation by nuclear hormone receptor. *Science*, **293**, 853-857.

Wang, Q., Stacy, T., Binder, M., Marin-Padilla, M., Sharpe, A.H. and Speck, N.A. (1996) Disruption of the Cbfa2 gene causes necrosis and hemorrhaging in the central nervous system and blocks definitive hematopoiesis. *Proc Natl Acad Sci U S A*, **93**, 3444-3449.

Watson, K.L., Johnson, T.K. and Denell, R.E. (1991) Lethal(1) aberrant immune response mutations leading to melanotic tumor formation in Drosophila melanogaster. *Dev Genet*, **12**, 173-187.

Wilder, E.L. and Perrimon, N. (1995) Dual functions of wingless in the Drosophila leg imaginal disc. *Development*, **121**, 477-488.

Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J. and Campbell, K.H. (1997) Viable offspring derived from fetal and adult mammalian cells. *Nature*, **385**, 810-813.

Winston, F. and Allis, C.D. (1999) The bromodomain: a chromatin-targeting module? *Nat Struct Biol*, **6**, 601-604. java/Propub/structbio/nsb0799_0601.fulltext java/Propub/structbio/nsb0799_0601.abstract.

Wittwer, F., van der Straten, A., Keleman, K., Dickson, B.J. and Hafen, E. (2001) Lilliputian: an AF4/FMR2-related protein that controls cell identity and cell growth. *Development*, **128**, 791-800.

Wodarz, A. (2000) Tumor suppressors: linking cell polarity and growth control. *Curr Biol*, **10**, R624-626.

Xu, W., Chen, H., Du, K., Asahara, H., Tini, M., Emerson, B.M., Montminy, M. and Evans, R.M. (2001) A transcriptional switch mediated by cofactor methylation. *Science*, **294**, 2507-2511.

Xue, Y., Wong, J., Moreno, G.T., Young, M.K., Cote, J. and Wang, W. (1998) NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. *Mol Cell*, **2**, 851-861.

Yamamoto, Y., Girard, F., Bello, B., Affolter, M. and Gehring, W.J. (1997) The cramped gene of Drosophila is a member of the Polycomb-group, and interacts with mus209, the gene encoding Proliferating Cell Nuclear Antigen. *Development*, **124**, 3385-3394.

Yang, X.J., Ogryzko, V.V., Nishikawa, J., Howard, B.H. and Nakatani, Y. (1996) A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature*, **382**, 319-324.

Yano, T., Nakamura, T., Blechman, J., Sorio, C., Dang, C.V., Geiger, B. and Canaani, E. (1997) Nuclear punctate distribution of ALL-1 is conferred by distinct elements at the N terminus of the protein. *Proc Natl Acad Sci U S A*, **94**, 7286-7291.

Yao, D.L., Webster, H.D., Hudson, L.D., Brenner, M., Liu, D.S., Escobar, A.I. and Komoly, S. (1994) Concentric sclerosis (Balo): morphometric and in situ hybridization study of lesions in six patients. *Ann Neurol*, **35**, 18-30.

Yu, B.D., Hanson, R.D., Hess, J.L., Horning, S.E. and Korsmeyer, S.J. (1998) MLL, a mammalian trithorax-group gene, functions as a transcriptional maintenance factor in morphogenesis. *Proc Natl Acad Sci U S A*, **95**, 10632-10636.

Yu, B.D., Hess, J.L., Horning, S.E., Brown, G.A. and Korsmeyer, S.J. (1995) Altered Hox expression and segmental identity in Mll-mutant mice. *Nature*, **378**, 505-508.

Yuge, M., Nagai, H., Uchida, T., Murate, T., Hayashi, Y., Hotta, T., Saito, H. and Kinoshita, T. (2000) HSNF5/INI1 gene mutations in lymphoid malignancy. *Cancer Genet Cytogenet*, **122**, 37-42.

Zeleznik-Le, N.J., Harden, A.M. and Rowley, J.D. (1994) 11q23 translocations split the "AT-hook" cruciform DNA-binding region and the transcriptional repression domain from the activation domain of the mixed-lineage leukemia (MLL) gene. *Proc Natl Acad Sci U S A*, **91**, 10610-10614.

Zhang, H.S., Gavin, M., Dahiya, A., Postigo, A.A., Ma, D., Luo, R.X., Harbour, J.W. and Dean, D.C. (2000) Exit from G1 and S phase of the cell cycle is regulated by repressor complexes containing HDAC-Rb-hSWI/SNF and Rb-hSWI/SNF. *Cell*, **101**, 79-89.

Zhang Y, Buchholz F, Muyrers JP, Stewart AF. (1998) A new logic for DNA engineering using recombination in Escherichia coli. *Nat Genet.*, **20**, 123-8.

Zhao, K., Kas, E., Gonzalez, E. and Laemmli, U.K. (1993) SAR-dependent mobilization of histone H1 by HMG-I/Y in vitro: HMG-I/Y is enriched in H1-depleted chromatin. *Embo J*, **12**, 3237-3247.

Zhou, X., Richon, V.M., Wang, A.H., Yang, X.J., Rifkind, R.A. and Marks, P.A. (2000) Histone deacetylase 4 associates with extracellular signal-regulated kinases 1 and 2, and its cellular localization is regulated by oncogenic Ras. *Proc Natl Acad Sci U S A*, **97**, 14329-14333.

Zink, B. and Paro, R. (1989) In vivo binding pattern of a trans-regulator of homoeotic genes in Drosophila melanogaster. *Nature*, **337**, 468-471.

Zink, D. and Paro, R. (1995) Drosophila Polycomb-group regulated chromatin inhibits the accessibility of a trans-activator to its target DNA. *Embo J*, **14**, 5660-5671.